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Analytical characterization of natural polymers during mashing processes and its influence on the palate fullness of beer

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To my sons

Acknowledgement

This thesis is the result of several years of work. It was carried out with the intention of making a contribution to a fascinating research area which is of interest throughout the world. What was scientifically achieved as a result of these years of work is described in the thesis. But what I learnt along the process, as a researcher and as a person is not. All the accomplishments that were made during these years of hard work, both the ones that are described in the thesis and those that are not, were possible thanks to the contribution of many people. To all of them I am very grateful.

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Preface and peer reviewed publications

The results and publications of this thesis were developed at the Technische Universität München, Institute of Brewing and Beverage Technology: from 2012 to 2017 under the supervision of Prof. Dr. Tomas Becker.

The following **peer reviewed publications** (shown in chronological order) were generated in the period of this work (publications which are part of this thesis are indicated in bold).

1. **Rübsam H, Krottenthaler M, Gastl M, Becker T. An overview of separation methods in starch analysis: the importance of size exclusion chromatography and field flow fractionation. Starch/Staerke 2012; 64: 683–695.**

2. Rühmkorf C, Rübsam H, Becker T, BorK C, Voiges K, Mischnick P,Brandt M, Vogel R. Effect of structurally different microbial homoexopolysaccharides on the quality of gluten-free bread. Eur. Food Res. Technol. 2012; 235: 139–146.

3. Rühmkorf C, Bork C, Mischnick P, Rübsam H, Becker T, Vogel RF. Identification of *Lactobacillus curvatus* TMW 1.624 dextransucrase and comparative characterization with *Lactobacillus reuteri* TMW 1.106 and *Lactobacillus animalis* TMW 1.971 dextransucrases. Food Microbiology 2013; 34: 52-61.

4. **Rübsam H, Gastl M, Becker T. Influence of the range of molecular weight distribution of beer components on the intensity of palate fullness. *Eur. Food Res. Technol*. 2013; 236: 65-75**.

5. Jakob F, Pfaff A, Novoa-Carballal, R, Rübsam H, Becker T, and Vogel RF. Structural analysis of fructans produced by acetic acid bacteria reveals a relation to hydrocolloid function. Carbohydr Polym. 2013; 92: 1234–1242.

6. **Rübsam H, Gastl M, Becker T. Determination of the influence of starch sources and mashing procedures on the range of the molecular weight distribution of beer using Flow Field Flow Fractionation. Jnl Institute Brewing 2013; 119: 139–148.**

7. **Rübsam H, Gastl M, Becker T. Analytical characterization of the hydrolysis of barley malt macromolecules during enzymatic degradation over time using AF4/MALS/RI. Journal of Food Science 2017; 82: 1326-1332.**

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Notation

Abbrevations

Symbol	Unit	Meaning/Definition
ADF	%	apparent degree of fermentation
AF4	-	asymmetric Flow Field-Flow Fractionation
BPB	-	bromophenol blue
CL	glucose units	chain length
D	-	diffusion coefficients
DE	-	dextrose equivalent
DP	-	diastatic power
DPn	-	number average degree of polymerization
DRI	-	differential refractive index
FFF	-	field flow fractionation
HDC-SEC	-	Hydrodynamic and Size-Exclusion Chromatography
HPAEC		high performance anion-exchange chromatography
HPSEC	-	High Pressure Size Exclusion Chromatography
KI	%	Kolbach index
M	-	molar mass
MALLS	-	multiple-angle laser light scattering
MALS	-	multi-angle light scattering
Mw	g/mol	weight average molar mass
MWD	kDa	molecular weight distribution
NC	glucose units	average number of chains per molecule
P	-	p-value / probability
PSD	-	Particle size distributions
Rg	Nm	radius of gyration
Rh	-	hydrodynamic radii
RI	Mw	Refractive Index detector
Rrms	Nm	radius of gyration
SEC	-	size-exclusion chromatography

Summary

The proportion and structure of beer macromolecules influence the mouthfeel of the beer. Asymmetric Flow Field-Flow Fractionation (AF4) has emerged as a powerful tool for the separation of complex biopolymers. In this work coupling AF4 to online multi-angle light scattering (MALS) with refractive index detectors allowed the determination of molar mass and molar mass distributions of macromolecules enabling the analysis of biopolymers in their native state at any point of the brewing process with no requirement for additional sample preparation steps.

Combining AF4/MALS/RI with sensory analysis it was possible to determine the effect of the overall molecular weight distribution (MWD) of the beer components on the intensity of the palate fullness. Thereby, the range of the MWD of different commercial pilsner beers, experimental beers as well as of different types of maltodextrins dissolved in samples of the same type of pilsner beer (spiking trial) was determined by means of AF4/MALS/RI. The intensity of the palate fullness of the different commercial and experimental pilsner beers as well as of the spiking trial was determined by sensory analysis carried out by a trained tasting panel. Also the threshold concentration, defined as the concentration value by which the palate fullness was perceived as different with respect to the control pilsner beer (without maltodextrin), of the samples of the spiking trial was determined. The results obtained confirmed that the palate fullness differed strongly among commercial beers. It was demonstrated that these variations were significantly associated to differences on the range of the MWD of the beer components. The range of MWD of the commercial pilsner beers corresponded to those found for commercial maltodextrins with intermediated range of MWD. The MWD of the maltodextrin in samples of the spiking trial varied noticeably according to the type of maltodextrin added to the beer sample. It was also found that the effect of the maltodextrin concentration on the intensity of the palate fullness was influenced by the range of MWD of the maltodextrins. However, this effect could be appreciated only when maltodextrins were added at concentrations between 5–20 g/L to the beer samples. Thus it was demonstrated that the intensity of the palate fullness was influenced by the range of the MWD of the beer components highlighting the contribution of maltodextrins.

It was also proved that modification of technological parameters during mashing influenced the MWD of the produced beer and so the beer palate fullness. The data obtained showed that the overall MWD of the beers was modified according to the initial temperature and the quality of the malted barley, resulting in beers with different palate fullness. AF4/MALS/RI analysis was also used to evaluate the influence of the type of mashing (infusion or decoction), as well as variations of mashing parameters (temperature and rest time) and the use of different adjuncts (pre-cooked maize and non-malted spelt) on the range of the MWD of the produced beers. The results showed that the range of the MWD of the beers obtained was not significantly affected by the type of mashing. However it was strongly influenced by the type of adjunct and the initial mashing temperature indicating the usefulness of the range of the MWD as a parameter to verify the type of beer produced under different mashing conditions.

Monitoring of the changes of MWD and particle size distributions of barley malt macromolecules that take place during starch amylolysis in mashing procedures could be carried out by means of AF4/MALS/RI. For this purpose isothermal mash/trials were performed according to amylolytic starch degradation processes. Therefore, temperatures were selected according to the corresponding α- and β-amylases range of activity (65 to 75°C). The samples were produced tracking amylolytic processes at different periods of time (1-90 min) in each mash/trial. The results showed that the selection of a particular temperature/time profile during mashing influences the molecular structure of starch degradation products. Thereby it was confirmed that macromolecular structure of the beer components will be modified by technological parameters during brewing which would affect the palate fullness of the produced beer.

Zusammenfassung

Der Anteil und die Struktur der Biermakromoleküle beeinflussen das Mundgefühl des Bieres. Asymmetric Flow Field-Flow Fractionation (AF4) hat sich als leistungsstarkes Instrument zur Trennung komplexer Biopolymere herausgestellt. Die Kopplung von AF4 und online multi-angle light scattering (MALS) mit Brechungsindexdetektoren ermöglichte in dieser Arbeit die Bestimmung der Molmasse und der Molmassenverteilung von Makromolekülen. Dadurch war die Analyse von Makromolekülen in ihrem ursprünglichen Zustand zu jedem Zeitpunkt des Brauprozesses ohne zusätzliche Probenvorbereitungsschritte möglich.

Durch die Kombination von AF4/MALS/RI und sensorischen Analysen/Untersuchungen wurde der Einfluss der Molekulargewichtsverteilung der Bierkomponenten auf die Intensität der Vollmundigkeit vom Bier ermittelt. Die Molekulargewichtsverteilung verschiedener Pils-Biere, Versuchsbiere sowie verschiedener Maltodextrinsorten (Spiking-Versuche) wurde mittels AF4/MALS/RI analysiert. Die Intensität der Vollmundigkeit der verschiedenen kommerziellen und experimentellen Pils-Biere sowie der Spiking-Versuche wurde mittels mehrerer sensorischen Analysen von einem geschulten Verkostungspanel festgestellt. Auch die Konzentration der Gescmacksschwellen der im Bier zugesetzen Maltodextrinen (Spiking-Versuche) wurde dabei ermittelt. Die erzielten Ergebnisse bestätigten, dass die Vollmundigkeit sich stark unter den kommerziellen Bieren unterschied. Hierbei zeigte sich ein eindeutiger Zusammenhang dieser Abweichungen mit den ermittelten unterschiedlichen Bereichen der Molekulargewichtsverteilung von den Bierkomponenten. Die Molekulargewichtsverteilung der Spiking-Versuche in Bierproben variierte signifikant je nach Art der zugesetzten komerziellen Maltodextrine. Es wurde auch festgestellt, dass die Wirkung der Maltodextrinkonzentration auf die Intensität der Vollmundigkeit durch den Bereich der Molekulargewichtsverteilung beeinflusst wurde. Dieser Effekt war jedoch nur zu erkennen, wenn Maltodextrine in Konzentrationen zwischen 5 und 20 g/l den Bierproben zugesetzt wurden. Somit zeigten diese Ergebnisse, dass die Intensität der Vollmundigkeit durch den Bereich der Molekulargewichtsverteilung der Bierkomponenten stark beeinflusst wird.

Es wurde nachgewiesen, dass Anpassungen von technologischen Parametern während des Maischens die Molekulargewichtsverteilung des produzierten Bieres und somit die Vollmundigkeit direckt beeinflussen können. Die erhaltenen Daten zeigten, dass die Molekulargewichtsverteilung der Biere durch die Variation der Einmaischtemperatur und die Qualität des angewendeten Gerstenmalzes auch modifiziert werden kann, was zu Bieren mit unterschiedlicher Vollmundigkeit führt.

AF4/MALS/RI wurde auch verwendet, um den Einfluss des Maischprozesses (Infusion oder Dekoktion) und der Anwendung anderer Rohstoffe (vorgekochtes Maismehl) auf die Molekulargewicstverteilung des fertigen Bieres zu bestimmen. Die Ergebnisse zeigten, dass der Bereich der Molekulargewichtsverteilungdaraus resultierten Biere durch die Art des gewählten Maischprozesses (Dekoktion oder Infusion) nicht signifikant beeinflusst wurde. Auf der anderen Seite zeigte sich die Einmaischtemperatur als wichtigster Parameter zu beeinflüssen die Molekulargewichtsverteilung des finalen Bieres durch den Prozess.

Die Überwachung der Variation der Molekulargewichtsverteilung und der Partikelgrößenverteilung von Gerstenmalzmakromolekülen, die während der Stärkeamylolyse bei Maischen stattfinden, konnte mittels AF4/MALS/RI erfolgreich durchgeführt werden. Zu diesem Zweck wurden isotherme Maischversuche bei Temperaturen von 65 °C bis 75 °C (gemäß der Aktivität der α- and β-Amylase) für 1 bis 90 Minuten durchgeführt. Die daraus erzielten Ergebnissen zeigten, dass die Auswahl eines bestimmten Temperatur/Zeit-Profils während des Maischens die Molekülstruktur der finalen Stärke-Abbauprodukte stark beeinflusst.

1 **Introduction**

Beer is a fermented beverage mainly composed by water, ethanol, non-fermentable carbohydrates and a small quantity of proteins. The technology of beer production began six thousand years ago by the world's oldest civilizations such as the ancient Egyptians and Mesopotamians (Cabras and Higgins, 2016). Nowadays, brewing is one of the lead food industries in the world, being beer production around 1.95 billion hectoliters (Worldwide Beer Production, 2017 Statistic.). Malted barley is the most used source of carbohydrates for brewing due to its appropriate level of protein, low level of lipids and the generation of high levels of amylolytic enzymes activity during germination. Degradation of barley malt by enzymes during mashing leads to a diversity of polymeric compounds that include proteins, polyphenols and polysaccharides. The proportion and structure of these macromolecules in beer determines the flavor and textural attributes of the beer. Thereby, characterization of these polymeric compounds makes necessary the development of new analytical approaches for the optimization of brewing processes in the industry.

1.1 Characterization of starch molecules

Starch is one of the most important polymers in nature. It constitutes the major energy reserve for higher plants such as cereals, legumes, tubers, rhizomes, unripe fruits, etc. It is the primary component of barley grains which are the main used material in brewing.

Starch consists of a semi crystalline granular structure composed basically by amylose, a linear polysaccharide containing α-1-4 glycosidic linkages, and amylopectin which is highly branched and contains α-1, 4 and α-1, 6 glycosidic linkages chains. The amylose/amylopectin ratio as well as their macromolecular structure determines physical and chemical properties such as viscosity, shear resistance, gelatinization, textures, solubility, gel stability, cold swelling, and retrogradation. These properties influence specific characteristics of cereal based food including bread, pasta, tortillas and beverages as well as of cereals that are consumed directly (for example, cooked rice, potato, corn, beans, etc.). Thus, accurate characterization of starch from different botanical sources has become a relevant issue to obtain commercial products meeting specific functional and sensorial specifications. Characterization of the molecular weight, molecular weight distributions, radius of gyration and size distributions of starch polymers have provided information that can be valuable to determine their functional and rheological properties as well as sensorial attributes of cereal based processed food (Aparicio-Saguilán et al., 2014; Bello-Pérez et al., 2017; Jakob et al., 2013; Jiménez et al., 2010; Morante et al., 2015; Rühmkorf et al., 2012) and beverages (Tügel et al. 2015; Zielke, Texeira, et al. 2017; Krebs, Becker, and Gastl, 2017).

Size separation techniques such as size-exclusion chromatography (SEC) and Asymmetric Flow Field-Flow Fractionation (AF4) coupled to multiple detection systems like differential refractive index, multiple-angle light scattering and in-line viscometry have provided data to elucidate the structure of complex polymers (Wahlund 2000; Cave et al. 2009; Gidley et al. 2010; Yohannes et al. 2011; Rübsam et al. 2012; Harding, Adams, and Gillis 2016). However, application of SEC to large polysaccharides involves a high risk of shear degradation due to the fine porous structure of the column packing (Cave et al., 2009). Due to its open channel geometry and the absence of a stationary phase AF4 has a number of advantages over SEC. AF4 enables separation of macromolecules at a very wide size range (from 1 nm up to 50 mm) favoring maximal sample recovery and analysis to be achieved in a wide range of aqueous solutions. On the other hand shear degradation is minimized helping analysis of sensitive and fragile analytes such as protein aggregates, macromolecular assemblies etc. (Wahlund 2000; Yohannes et al. 2011).

1.2 Principle of Asymmetrical Flow Field Flow Fractionation (AF4)

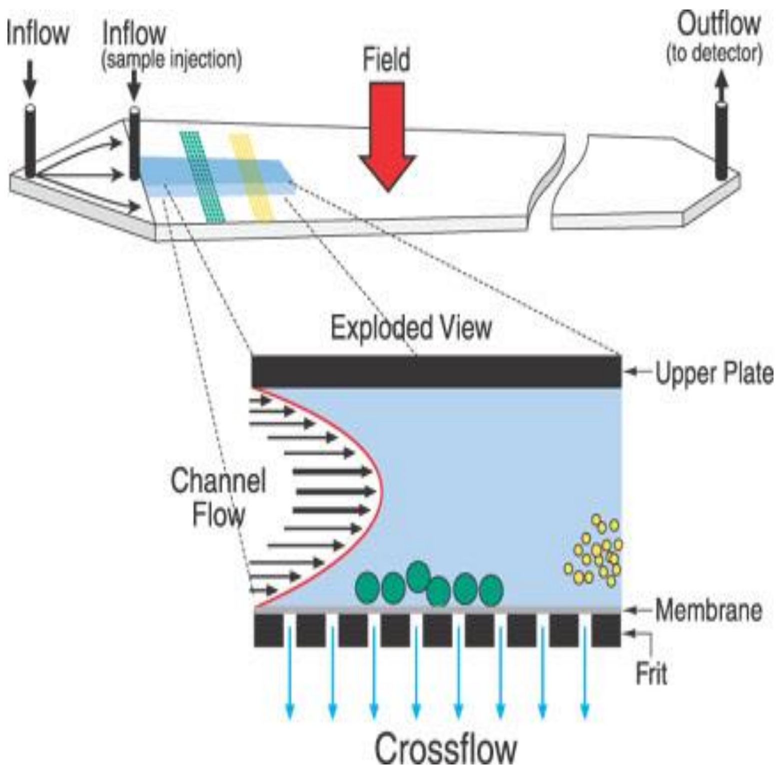
The concept of field flow fractionation (FFF) was presented by Giddings (Giddings, 1993). Since then, various sub-techniques of FFF have been developed. They include sedimentation FFF, thermal FFF, electric FFF, and flow FFF. In all cases, sample separation is carried out inside a narrow “ribbon-like” channel. Flow field flow fractionation (F4), introduced in 1976 has proven to be the most universal and most frequently used of all FFF techniques (Schimpf et al., 2000). This is because the technique employs a hydrodynamic field applied by means of a secondary flow (cross flow) of carrier liquid, perpendicular to the main flow (Rübsam et al., 2012; Schimpf et al., 2000; Wahlund, 2000; Yohannes et al., 2011). Thus, when pumping a bulk liquid into the channel a convective flux is generated. The liquid then exits the channel through the opposite wall, the so called “accumulation wall”, that consists of a membrane placed on the top of a porous wall. For this reason, retention time in F4 is merely dependent on diffusive flux and the separation of macromolecules or particles occurs according to their diffusion coefficients (Yohannes et al., 2011).

There are two main types of flow FFF: symmetrical flow FFF and asymmetrical flow FFF.

The symmetrical flow FFF channel consists of upper and lower semi-permeable porous frits within the external blocks. When sample materials are introduced to a symmetrical flow FFF channel, they are pushed towards one side of channel wall (accumulation wall) by the applied field (cross flow). The main channel flow is stopped during the relaxation period for the time needed for the cross flow pump to deliver about one channel volume. During this short period of time, sample components find equilibrium positions where the field force and the diffusion are counterbalanced, and the sample components are differentially distributed along the channel cross section/thickness according to their sizes (Yohannes et al., 2011).

In the asymmetrical flow field flow fractionation (AF4) the upper wall of the symmetrical flow FFF channel is replaced by an impermeable glass plate. The bottom channel plate is permeable, and made of a porous frit material. AF4 was first introduced in 1987 (Wahlund and Giddings 1987) and optimized further in the beginning of nineties (Litzén et al., 1993).

Details about the AF4 approach have been reviewed elsewhere (Qureshi and Kok, 2011; Rübsam et al., 2012; Yohannes et al., 2011). AF4 is carried out on successive phases as shown in Figure 1.



Separation of macromolecules and particles at a range size about 1 nm to 50 microns or polymers of about 10^3 Da to 10^{12} Da.

The channel consists of two plates:
The upper limit: solid impermeable wall
The lower limit: equipped with a porous frit and a suitable membrane

The cross flow is generated directly inside the channel, where the main flow coming from the inlet is divided to generate a cross flow through a semi-permeable membrane that is located on the bottom wall, while the rest of the longitudinal flow stream is directed to the detector system .

Figure 1: Asymmetric Flow Field Flow Fractionation (AF4) scheme.
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During the first phase, the flow is directed to enter the channel via the inlet, the only outlet being through the membrane. The sample is introduced into the channel during this phase and focused at the position in the channel determined by the counteracting flows, where the axial velocity is zero. Sample components move to equilibrium height over the membrane. The next phase is elution, where the flow enters the channel from the inlet and leaves both through the membrane (creating the crossflow) and through the channel outlet (Figure1). The carrier flow is laminar into the channel, so the transportation speed of the sample is correlated to its distance from the membrane wall. The interplay between field-induced migration toward this membrane and Brownian motion away from the wall leads to an exponential decrease with increasing distance from the membrane. This corresponds to the normal mode operation under which small particles are eluted first (Figure1).

The migration velocity of an analyte down the channel (and thus the retention time) is determined by its diffusion coefficient. As the diffusion coefficient increases, the migration velocity increases, yielding separation of components according to their diffusion coefficients (Cölfen and Antonietti, 2007; Wahlund, 2000; Yohannes et al., 2011).

Detector systems:

Aside from physically separating or fractionating the samples it is also important to consider the detection methods available. Three types of detectors are widely used: differential refractive index (DRI), multiple-angle laser light scattering (MALLS) and in-line viscometry.

Following separation by AF4, the samples are usually characterized by the use of light scattering in conjunction with Refractive Index detector (RI) which allows direct molar mass determination without calibration against molar mass standards (Wahlund 2000; Nilsson 2013). The most commonly light scattering detectors are multi-angle light scattering (MALS) which detect the scattered intensity at different angles in relation to the incoming light. The light scattering data obtained from the detector is fitted to different models of which the Debye, Zimm and Berry models are the most utilized (Nilsson, 2013). Basically, the models allow the determination of the rms radius (rrms) or radius of gyration (rg) from the slope of a curve fitted to the angular dependence of the intensity of scattered light. Provided that the refractive index increment with concentration (dn/dc) is known for the substance, and a RI detector is used, the molar mass (M), or more specifically the weight average molar mass (Mw) of each fraction can be determined from the intersection of the fitted curve. For large polymers, the Berry extrapolation method is better used because good accuracy is obtained for the molar mass and rrms without utilizing higher order polynomial fits (Rolland-Sabaté et al. 2007).

1.3 Application of AF4/MALS/RI to starch analysis

AF4 coupled to multiple detection systems has emerged as a powerful tool for obtaining data of hydrodynamic radius, radius of gyration, molar mass, polymer conformation, and branching degree of starch polymers (Rolland-Sabaté et al 2007). The reported values were close to those previously obtained by HPSEC and were in good agreement with previous data in the literature. Further, the effectiveness of AF4/MALS/RI was compared to that of Hydrodynamic and Size-Exclusion Chromatography (HDC-SEC) coupled with MALS or to online quasi-elastic light scattering, for the analysis of the structural properties of starch polymers from different starch sources (Rolland-Sabaté et al., 2011). It was demonstrated AF4 enabled a better separation of amylopectins than HDC-SEC and a better structural characterization (hydrodynamic radius distributions and molar mass distributions) of the polymers (Rolland-Sabaté et al., 2011). The technique has also been successful to compare the structure of starch polymers from different botanical sources varying on their amylose/amylopectin radio (Chiaramonte et al., 2012; Wahlund et al., 2011)**.**

There are also many reports indicating that AF4/MALS/RI can be used to characterize starch macromolecules from botanical sources with potential for food and beverage industry. For example, determinations of the weight-average molar mass and radius of gyration has contribute to the identification of new natural sources of amylose free starch from “Mapuey” tubers (*Dioscorea trifida*) which is an important staple food in the Caribbean and Amazon regions (Jiménez et al., 2010)**.** More recently the tool has been used for the characterization of amylopectin and amylose of other aroid starches with commercial importance in Africa, Asia, Central and South America (Hoyos-Leyva et al., 2017). Structural properties from cassava, that after maize is the second most important source of production of commercial starch worldwide, were also determined using AF4/MALS/RI (Morante et al., 2015). In addition, a combined approach using AF4/MALS/ RI and AF4 coupled to a fluorescence detector had facilitated the study of the interaction between proteinaceous material and polysaccharide in starch samples (Han’guk Punsŏk Kwahakhoe. et al., 2017). Application of this approach allowed the study of β-glucan molecular structure including the presence of proteinacious moeitys covalently bound to β-glucan chains from purified extracts from oats and barley. For this purpose extracts were labeled in-line, post-channel with Calcofluor to detect β-glucan or pre-injected with EDAC to label peptide bonds, and then analyzed using the AF4-MALS-FL set-up (Zielke et al. 2017).

The use of AF4/MALS/RI or AF4/ MALS/ dRI/UV for the analysis of the structural properties of beer has begun to succeed in the last years (H Rübsam, Gastl, and Becker 2013; Tügel et al. 2015; Krebs, Becker, and Gastl 2017; Choi et al. 2017; Rübsam, Becker, and Gastl 2017). The possibility to identify structural properties of beer components influencing beer sensorial attributes certainly will provide of a potential technological tool for commercial applications in cereal-based beverages production.

1.4 Beer production

1.4.1 Starch sources

Barley *Hordeum vulgare,* a grass belonging to the family *Poaceae* is the most source of starch used in brewing. Malted Barley has an appropriate level of protein (8 – 12% dry weight), low level of lipids and high levels of amylolytic enzymes produced during germination. It has a husk (the outer case of the barley grain) that creates a filter bed which may facilitate separation of insoluble material from the extract (Briggs et al. 2004; Narziß 2005; Bamforth and American Society of Brewing Chemists. 2006; Wunderlich and Back 2009). Other cereals, including wheat, rice, maize, oats and sorghum are used as adjuncts for certain beers (Bamforth 2009). Their application is regulated in every country. In Germany, the use of malt substitutes is prohibited according to the *Reinheitsgebot* (the Purity Law governing commercial brewing). The United States allows an input of unmalted cereals of up to 34% and an input of sugars or syrups of up to 2.5% of the total grist.

The hull or husk constitutes around 10% of the dry weight of barley grain. The hull is rich in cellulose, arabinoxylans, lignin, polyphenols and minerals. The lemma and palea of the hull covers the caryopsis (kernel) which includes pericarp, seed coat and endosperm. The pericarp contains cellulose, lignin and arabinoxylan. The endosperm, which contributes to 75% to 80% of the total kernel weight, is rich in starch which is embedded in a protein matrix (Vasanthan and Hoover, 2009). Cell walls of barley endosperm exhibit a layered structure where the outer cell wall is made up of arabynoxylans, ferulic and acetic acids and the inner side mainly by β-glucan molecules (Kanauchi and Bamforth 2002; Bamforth 2017). Thus, enzymatic hydrolysis of the arabinoxylan layer allows better access of glucanases during malting, preventing disadvantageous effects of an excess of β-glucans on the malt extract such as viscosity or/and filtration speed (Fox 2008; Steiner, Procopio, and Becker 2015).

Starch is the most abundant component of the endosperm, comprising around 60% of total grain weight. It consists of approximately 75% amylopectin and 25% amylose. Although built up from the same glucopyranose units, these polymers differ in the type of glycosidic linkages and require different enzymes for their degradation. Amylopectin is a very large, branched molecule made up of glucose units linked by α-1,4 bonds (which give linear chains) and glucose units linked by α-1,6 bonds (which give branch points). On average, each branch is made up of around 25 glucose units. Amylose, on the other hand, is a linear molecule made up of up to 2,000–5,000 glucose units linked by α-1,4 bonds only (Buléon et al. 1998; Singh et al. 2003; Tester, Karkalas, and Qi 2004; Copeland et al. 2009; Vasanthan and Hoover 2009). Structure of starch granule is detailed in Chapter 1 and schematized in figure 2.

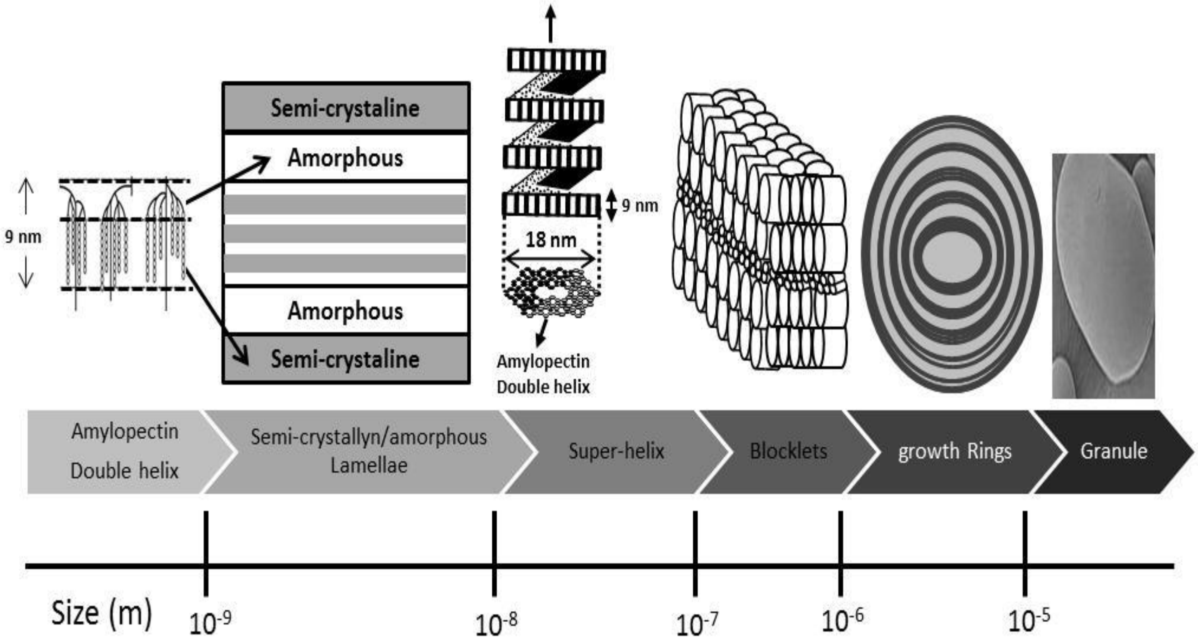

Figure 2: Starch granules are formed by alternating semi-crystalline and amorphous shells named growth rings, ranging from 120 to 400 nm in thickness, which extend from the center of growth towards the surface of granules. The semicrystalline shells are formed by the association of several blocklets which are thought to be spherical structures (20–250 nm) formed by units of associated semi crystalline lamellae which consists of repeat units of crystalline lamellae alternating with amorphous lamellae with a periodicity of approximately 9 nm. The crystalline lamellae are assumed to be formed by clusters of AP short chains (12–20 glucose units) joined by longer chains (40 or more glucose units), which span two or three more clusters Adjacent short chains within the clusters form left handed helices that pack together in a tetragonal array leading to the crystalline structure of the lamellae. The branching points may be located within the amorphous Lamellae. The crystalline lamellae may form a more or less continuous super helical structure with a diameter of approximately 18 nm and a pitch of 10 nm. A central cavity within the super helices would have a diameter of about 8 nm. Location of AM within the structure of the granule is still not well elucidated but may be located as individual chains within the amorphous zone of the growth rings.
Adapted from: Rübsam H, Krottenthaler M, Gastl M, Becker T. An overview of separation methods in starch analysis: the importance of size exclusion chromatography and field flow fractionation. Starch/Staerke 2012; 64: 683–695. The granule picture at the right of the scheme is a courtesy of the Institute of Brewing and Beverage Technology, TUM-Weihenstephan.

Barley starch granules consist mainly of a mixture of large lenticular A granules (10 to 30 µm) and smaller irregularly shaped (6 µm) B granules. The large granules constitute 10 to 20% of the total number of starch granules and around 85% of the weight of total starch mass. The small granules constitute 85% by number and around 15% by weight. Barley starches generally exhibit an A-type x-ray pattern which is characteristic of cereal starches. However, certain barley cultivars exhibit a mixed A/B-type pattern which is characteristic of legume starches (Vasanthan and Hoover, 2009).

The amylose content of barley starches is up to 46%. Structural characteristics of barley amylose has been determined (Song and Jane, 2000; Takeda et al., 1999); being the number average degree of polymerization (DPn) of 940 to 1000, the average chain length (CL) of 115-530 glucose units, the average number of chains per molecule (NC) of 6-10 glucose units, the weight average molecular weight (Mw) varies from 2.73 x 10^6 to 5.67 x 10^6 g/mol and the radius of gyration (rms) from 64 to 148 nm whereas the β-amylolysis limit, iodine affinity and limiting viscosity number are in the range of 70% to 95%, 17.4 to 20% and 240 to 391 g/ml, respectively; also barley amylose is complexed to a lysophosphatidyl choline in a ratio of 1:7.

Debranched amylopectins of normal, waxy and high-amylose barley starches exhibit similar chain length distribution profiles, all showing trimodal distributions of short chains (mostly A-chains), intermediate chains (mostly B 1 – and some A-chains) and long chains (mostly B 2 and B 3 chains) (Vasanthan and Hoover, 2009). The average chain length and the degree of branching are in the range of 17.6 to 22 glucose units and 4.2 to 5.7 glucose units respectively (Naguleswaran et al., 2014; Song and Jane, 2000; Yoshimoto et al., 2002; You and Izydorczyk, 2002). The weight average molecular weight of normal barley amylopectin has been reported to be around 22.4 x 10^6 g/mol whereas that the average radius of gyration (rs) of normal barley amylopectin is around 71.9 nm (Naguleswaran et al., 2014). The molecular characteristics of amylopectin such as molar mass (molecular weight), molecular dimension or size (radius of gyration), molecular density, branching degree, and distribution of short-chains have been shown to influence starch hydrolysis by amylases (Naguleswaran et al., 2014). Proteins, lipids and phosphorus are the minor non-carbohydrate components of barley starch. Integral proteins have been shown to be present in the central and peripheral regions of the granule (Rittenauer et al., 2016; Vasanthan and Hoover, 2009). Starch lipids are present on the granule surface and interior. The internal lipid content of small barley starch granules is higher than that of large granules and is proportional to the amylose content (Tester, 1997; You and Izydorczyk, 2002).

1.4.2 Starch degradation during brewing

The production of beer includes malting and brewing and every step has an impact on the quality properties of the resulting beer. There are four main steps during the brewing process which are wort preparation that includes mashing and boiling, fermentation, maturation, and filtration and/or stabilization. Comprehensive reviews of the brewing process can be found in the literature (Bamforth and American Society of Brewing Chemists. 2006; Bamforth 2017; Narziß et al. 2017). This work will focus on the process of starch degradation occurring during mashing procedures. Before mashing, malt has to be milled in a hammer mill to remove metals, dust and stones. Milling favor exposure of reactive surfaces for enzymes, making dissolution processes much easier. The quality of milling influences mashing and lautering processes and hence the quality of the resulting beer (Bamforth and American Society of Brewing Chemists. 2006; Palmer 2006; Wunderlich and Back 2009; Narziß et al. 2017). During mashing, grist is mixed with water. Temperature, rest time, pH-value, and water/ grist ratio are critical parameters determining enzymatic processes occurring during starch degradation processes (Evans et al. 2003; Celus, Brijs, and Delcour 2006; Gupta, Abu-Ghannam, and Gallaghar 2010; Bamforth 2017; Rittenauer, Gastl, and Becker 2017).

The dissolution of starch polymers takes place in three steps which are gelatinization, liquefaction and saccharification (Figure 3).

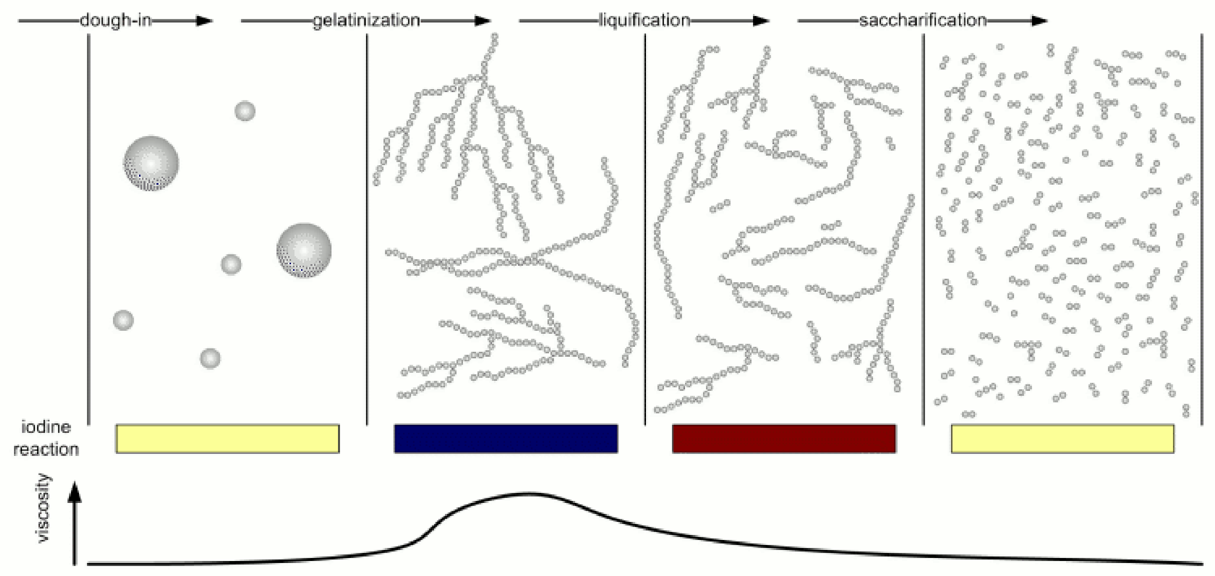

Figure 3: Schematic representation of starch dissolution during mashing processes. Adapted from Wolfgang Kunze, *Technologie Brauer und Maelzer*, 9. Auflage, VLB Berlin, 2017.

Gelatinization refers to the process, that take place during heating, in which starch loses the native order and crystalline structure of the granules and becomes amorphous (Ai and Jane, 2015). The gelatinization temperature is the specific temperature at which the starch granules in an aqueous suspension begin to swell and lose their native structure. It is influenced by characteristics of the starch granules: composition, morphology, molecular architecture, and molecular weight (Schirmer, Jekle, and Becker 2015; Rittenauer, Gastl, and Becker 2017). Barley starch gelatinizes at 61.3°C (Gujral et al., 2013; Schirmer et al., 2015). Other starch suppliers like rice, corn, rye or sorghum, exhibit different optimum temperatures for gelatinization (Briggs et al., 2004).

1.4.3 Starch amylolysis

The α-Amylase is an endo-acting enzyme that rapidly and randomly attacks the α-1,4-glucosidic linkages of starch (Figure 4) producing maltose, glucose, and α-limit dextrins, which contain the α-1,6-glucosidic linkages of the branched molecules of starch (Robyt, 2009). The action of α-amylase on starch produces rapid reduction of the extract viscosity and so it is called liquefying amylase. Attack is slower at the chain-ends and stops close to α-(1, 6) branch points. The products of extensive α-amylolysis include glucose, maltose, and a complex mixture of branched and unbranched oligosaccharides like maltotriose, maltopentaose and maltohexaose and maltoheptaose (Robyt, 2009). The α-amylase liberates the dextrins that are the substrate for the β-amylase. The β-Amylase is an exo-acting enzyme that cleaves the penultimate α-(1, 4)-link of the non-reducing chain ends of amylose and amylopectin (Figure 4), releasing the reducing disaccharide maltose which is the most abundant sugar in wort, but cannot hydrolyze the α–1,6-glucosidic linkages thus producing also β-limit dextrins (Robyt, 2009). The α–Glucosidase is also an exo-acting enzyme that cleaves non-reducing α–1,4-linkages liberating glucose. This amylase enzyme participates in starch hydrolysis mainly during the early stages of starch degradation in germinating barley seeds (Guerra et al., 2009; Sun and Henson, 1991). β-Amylase and α-glucosidase are known as saccharifying amylases. Limit dextrinase is an endo-acting enzyme that can attack the α-1,6-linkages (Figure 4) which are present in α and β-limit dextrins to allow their further hydrolysis by α-amylase (Robyt, 2009).

Joint activity of α-amylase, β-amylase, and limit dextrinase has been denominated as the diastatic power (DP) of the malt extract which in brewing is a critical parameter of malting quality, being an indicator of the capacity of the malt to degrade starch into fermentable sugars.

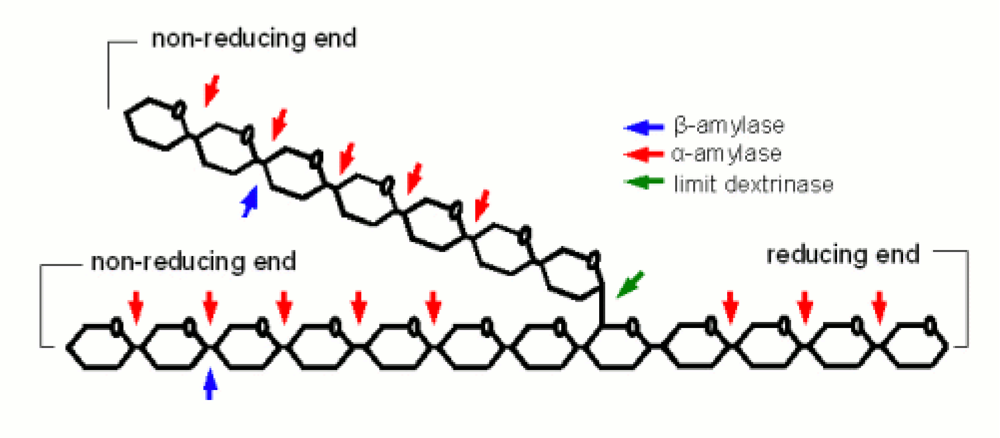

Figure 4: Sites of action of amylases involved in starch degradation. Adapted from braukaiser.com/images/Starch_conversion_enzyme.gif, 2018.

During mashing, barley starch hydrolysis is carried out mainly by α- and β-amylase. The activity of limit dextrin is usually low in malted cereals, making up less than 20% of its potential total activity due to the major presence of inactive forms of the enzyme which are bound to endogenous proteinaceous inhibitors (Bamforth 2009; Guerra et al. 2009). Starch hydrolysis must be completed to produce wort with an adequate composition of fermentable sugars which requires the use of malt with high DP. The levels and composition of fermentable sugars depend on the action of all the enzymes that are involved in starch degradation. Adequate levels of alpha-amylase, beta-amylase and limit-dextrinase enzymes are required for efficient conversion of starch to fermentable sugars during mashing. The levels of α and β amylases in the malt extract determine the percentage of starch hydrolyzed to fermentable sugars (MacGregor et al., 1999). Also, fermentable sugars in the mash liquor are positively correlated with the concentration of reducing sugars and the percentage of solubilized starch during mashing (MacGregor et al., 1999). Addition of limit-dextrinase to the mashes resulted in a substantial increase in levels of fermentable sugars, and limit dextrinase showed a synergistic effect in increasing levels of maltose in the mash liquor when combined with high levels of beta-amylase (MacGregor et al., 1999). Thus the efficiency of any one starch degrading enzyme in a mash is influenced by the presence of other enzymes. Extensive degradation of starch that occurs during mashing depends on the concerted action of the mixture of enzymes present and may be limited by enzyme destruction rather than by the absence of substrates for possible enzyme attack.

During mashing, temperature should be rise up to 61.3°C to ensure barley starch gelatinization for further enzymatic hydrolysis (Gujral et al., 2013; Källman et al., 2015). The rise on temperature can affect the activity of amylolytic enzymes in a different extent depending on their specific activities and on their resistant to thermal denaturation, also this effect may increase with longer mashing times. This way, an adequate amount and ratio of the enzymes could be suboptimal if the conditions of mashing are particularly inadequate for one of them (Guerra et al., 2009). Thus, α-amylase which is the most resistant enzyme to heat inactivation exhibits a higher optimal temperature around 70°C, whereas β-amylase and limit dextrinase show maximum enzymatic activities at 60°C to 62.5°C, Hence, an excess of β-amylase would be needed in malt to counterpart the loss of activity due to heat inactivation during mashing (MacGregor et al., 1999). However, achieving heat stability of β-amylase may be a better strategy to increase the levels of fermentable sugars in the wort. The goal of mashing is to reach the maximal fermentable carbohydrates productivity, while respecting a specification on the final non-fermentable dextrin concentration to ensure particular beverage attributes in the final beer (Buiatti S, 2009; L Narziß et al., 2017). Carbohydrates composition in the wort will depend on the make-up grist and the mashing conditions. Maltose and maltotriose are the major sugars in wort, but variable amounts of non-fermentable dextrins are also present. Maltose usually accounts for 50–60% of the fermentable sugar in wort (Ferreira, 1999). Carbohydrate fermentability of the wort is determined by the proportion of fermentable sugars and dextrins, usually the results are presented as fermentable carbohydrates as a percentage of the total carbohydrates.

1.5 Beer macromolecules

In addition to water and ethanol, beer contains a complex mixture of macromolecules derived as a result of the brewing process including fermentable sugars, dextrins remaining from enzymatic starch hydrolysis, polysaccharides compounds (β-glucans and arabinoxylans) coming from cell walls surrounding starchy endosperm of barley kernel and proteinaceous compounds (peptides and aminoacids). Other components such as phenolics compounds contribute to beer flavor, physical stability, and antioxidant activity.

Proteins:

The presence of low quantities of protein derived molecules in beer can contribute to foaming properties (Steiner, Gastl, and Becker 2011) and to the mouthfeel, influencing the palate fullness of the beer (Langstaff and Lewis, 1993). On the other hand, excess of proteins must be precipitated and removed from beer to prevent formation of haze (Steiner, Gastl, and Becker 2011).

Carbohydrates:

β-glucans and arabinoxylans

In addition to carbohydrates coming from starch amylolysis during mashing, small quantities of β-glucans and arabinoxylans from cell walls of starchy endosperm of barley kernel can be found in some types of beer. β-glucans in very low concentration can have a positive influence on beer smoothness and foam retention. However the presence of dissolved, high molar mass β-glucans increases the viscosity of wort causing turbidity which is a relevant quality failure (Steiner, Gastl, and Becker 2011). Thereby, the extent of degradation in barley cell wall polysaccharides plays an important role in the colloidal stability of beer.

Carbohydrates from starch hydrolysis:

Remnant carbohydrates from starch hydrolysis in beer include variable quantities of fermentable sugars which directly contribute to the sweetness of beer as well as of dextrins containing two or more branches (20–30 g/L in a pilsner beer) that have been associated to the palate fullness (Langstaff and Lewis, 1993). In fact carbohydrates in beer are mainly non-fermentable dextrins (90%) which cannot be metabolized by yeast strains. Usually the final beer will contain only low levels of fermentable sugars other than those added to sweeten some types of beer. Dextrins are branched polysaccharides including poly-disperse species. The most known are maltodextrins which are starch hydrolysates that consist of α-D-glucose units linked primarily by (1→4) glycosidic linkages with a dextrose equivalent (DE) of less than 20 (Kennedy et al., 1995). According to their DE value, maltodextrins have different physicochemical properties like solubility, freezing temperature and viscosity among others. However, maltodextrins with similar DE value exhibit very different properties depending on the hydrolysis procedure, botanical source of starch and amylose/ amylopectin ratio (Alvani et al., 2011; Chronakis, 1998; Kennedy et al., 1995).

1.5.1 Beer macromolecules influencing the palate fullness

The different combinations of ingredients, production processes, and storage conditions result in a great variety of beers, differing strongly in their chemical composition, and hence in their mouthfeel. Among the sensory attributes of the beer, the mouthfeel is as important to the sensory impact as appearance, aroma and taste. The mouthfeel comprise three key attributes which are carbonation, palate fullness and aftertaste

(Langstaff and Lewis, 1993). Carbonation is the first attribute perceived in the mouth, felt as a “tingle” due to the amount of carbon dioxide in the beer. The palate fullness is linked to the sensation of fullness or viscosity in the mouth, while it is being consumed. The final attribute of the mouthfeel is the aftertaste which comprises the last sensations recognized in the mouth such as stickiness, astringency, dryness and bitterness. The aftertaste in beer is mainly determined by the bitter that comes from the hops (Langstaff and Lewis, 1993; Magli et al., 2016).

There is evidence that polysaccharides and proteins in beer can affect the perception of palate fullness and so the mouthfeel. Proteinaceous compounds in beer are found in very low quantities. However, associations between protein concentration in beer with the palate fullness have been reported (Steiner, Gastl, and Becker 2011; Langstaff and Lewis 1993). On the other hand, there is evidence that unfermentable dextrins, also contribute to the palate fullness. Early studies (Louant and Dufour, 1991; Vermeylen, 1962) have shown a relationship between wort dextrin concentration and beer palate fullness. However, other authors reported that a very high concentration of (50 g/L) of maltodextrin needs to be added to a light beer to enhance significantly its palate fullness (Ragot et al., 1989). Because the natural content of dextrins in a beer ranges from 10 to 50 g/L, the authors concluded that dextrins alone do not account for the beer palate fullness. In contrast more recently, other authors have reported that addition of maltodextrins to low alcohol beers increase significantly the body and mouthfeel of the beer (Liguori et al., 2015). As discussed below, the results of this work showed that the effect of the maltodextrin concentration (5-20 g/L) on the intensity of the palate fullness was influenced by the range of MWD of the maltodextrins (Rübsam et al., 2013). In according, previous studies carried out to characterize beer components using LC-NMR/MS (Duarte et al., 2003) or ^{1}H NMR Spectroscopy (Barros et al., 2006) have demonstrated the presence of maltodextrins of different molar masses in the final beer which also may differ in their branching degree (Barros et al., 2006). These differences are due to the type of beer analyzed (Duarte et al., 2003) and of distinct brewing conditions that vary between breweries (Barros et al., 2006), which may account for the differences on the palate fullness usually perceived among distinct types of commercial beers.

However, it must be considered that perception of the palate fullness as a sensory attribute is influenced by many factors, so is difficult to associate the palate fullness to a particular beer component.

2 **Thesis outline**

Beer, a yeast-fermented beverage, is comprised of polymeric compounds that include proteins, polyphenols, and polysaccharides. These macromolecules, commonly characterized based on their molar weight and size distributions, can influence sensory quality parameters such as the palate fullness. Therefore, characterization of the MWD and size distributions of beer macromolecules can be relevant for the development of new technologies for the optimization of brewing processes in the industry. In this regard asymmetrical field flow fractionation (AF4), which has shown to be successful for the characterization of large and polydisperse polymers, would be suitable for the determination of molar mass distributions and size distributions of beer macromolecules. The approach allows the characterization of macromolecules in their native state with minimal sample preparation, thus during brewing samples could be analyzed at any point of the process with very little requirements. Therefore the following study questions were formulated:

Is AF4/ MALS/RI a suitable approach for beer analysis?

Is the molar mass distribution of beer components an effective analytical parameter for the evaluation of the beer palate fullness?

Do the maltodextrins present in the final beer influence the palate fullness?

Can technological parameters, such as the mashing regime and starch sources, influence the Molar Weight Distribution of beer polymers?

Can AF4/MALS/RI be used as a tool to track the changes of MWD and PSD distributions of barley malt carbohydrates occurring due to starch amylolysis along the mashing procedure?

Consequently, this study proposed the use of AF4/MALS/RI as an analytical approach to determine the molar mass distributions and particle size distributions of the beer as well as to evaluate the effect of different technological parameters of the brewing process on the molar mass and size distributions of beer components. Combining this tool with sensory analysis the effect of the molecular weight distribution of beer components on the palate fullness could be verified. Also, AF4/MALS/RI was used to study the effect of temperature on the changes of the structure of barley malt macromolecules occurring due to starch hydrolysis during the mashing process.

To achieve this goal the following research steps were developed:

1. **Determination of the effect of the MWD of beer components on the beer palate fullness**.

For this purpose, instrumental parameters of AF4/MALS/RI were standardized (choice of membrane, validation of channel performance, carrier liquids, injected amount and flow conditions) to determine the range of the MWD of different commercial pilsner beers. Also, to study the effect of maltodextrins on the beer palate fullness, the MWD of different types of commercial maltodextrins dissolved in samples of the same type of pilsner beer (spiking trial) were measured. The intensity of the palate fullness of the different commercial and experimental pilsner beers as well as of the spiking trial should be assessed by a trained tasting panel. Statistical associations between the MWD of the different beers and samples of the spiking trial with the palate fullness were determined.

2. **Effect of the modification of technological parameters (temperature and barley malt quality) during mashing on the MWD of the produced beer and its association with the palate fullness**.

To meet this objective, an amount of experimental beers were produced in the laboratory at a scale of 10 L. Malts with different Kolbach Index (41% and 36%) as well as different initial mashing temperatures (45°C, 55°C, 63°C) were tested while all further phases of the mashing process (boiling wort, wort filtration, fermentation, beer filtration and beer filling storage) were performed identical in all trials according to standard procedures. The MWD of the resulting beers was determined using AF4/MALS/RI (using the same AF4 method standardized for commercial beers) and the palate fullness for each bear sample was tested by sensory analysis. Statistical comparisons were carried out between the MWD of the distinct experimental beers and the palate fullness.

3. **Evaluation of the influence of the type of mashing as well as variations on the mashing parameters and the use of different adjuncts on the range of the MWD of the resulting experimental beers by using AF4/MALS/RI.**

Based on the previous results indicating the importance of the effect of the initial temperature and malt quality on the MWD and on the palate fullness of the beer a broader study was designed. Thereby, experimental beers were prepared with different mash mixtures and distinct mashing procedures (infusion and decoction), with variations of the rest time and initial temperatures were used. The range of molecular weight distribution (MWD) of the resulting beers was determined using AF4/MALS/RI. The procedures were carried out using barley malt with a Kolbach index (KI) of 41% as standard raw material. Additionally two different adjunct mixtures were used: barley malt mixed with commercial dry pre-cooked maize and barley malt mixed with non-malted spelt. For the standard barley malt as well as for the two adjunct mixtures, infusion or decoction (one and two steps) methods were used. Additionally in the infusion method, the effect of different initial mashing temperatures (45°C, 55°C and 63°C) and rest times (1 min, 15 min, 30 min) on the MWD of the resulting beers was determined. Also total nitrogen concentration and the apparent degree of fermentation was determined for each beer produced according to the selected mashing conditions. The MWD, total nitrogen concentration and ADF values were compared among beers produced under different mashing conditions. Also, in this research steep, a new protocol of AF4/MALS/RI was standardized (membrane size, time focusing and flow conditions) to measure the MWD and the radius of gyration (rms) of the mash samples (malted barley 41% KI, malted Barley mixed with pre-cooked maize as well as malted barley mixed with non- malted spelt). The conformation (rms vs molar mass) of the mix of macromolecules were determined and compared among the different types of mash samples (malted barley 41% KI, malted Barley mixed with pre-cooked maize and malted barley mixed with non- malted spelt).

4. **Determination of the changes of MWD and PSD distributions of barley malt macromolecules that take place during starch amylolysis in mashing procedures using AF4/MALS/RI.**

To meet this goal, AF4/MALS/RI analysis standardized previously for the analysis of barley malt samples, was used to monitor starch amylolysis in small scale-isothermal mashing trials. The approach was focused on amylolytic starch degradation and so mashing temperatures (65°C, 70°C and 75°C) were selected according to α- and β-amylases range of activity. Samples were produced by triplicate, tracking amylolytic processes over time periods from 10 to 90 min in each mash/trial.

3 **Results**

The publications containing the results of this work are detailed in the following chapters in the copies of the individual publications. The author made all the scientific experimental work responsible and was writing the publications as first author. Authors agreed with the data and information included taking responsibility over the content stating that there is no conflict of interest.

Chapter 1 Page 29-41	**An overview of separation methods in starch analysis: The importance of size exclusion chromatography and field flow fractionation.**

The purpose of this chapter was to gather information about the structural and functional properties relevant for the improvement of dissolution and degradation processes as well as to compare distinct separation techniques coupled with multiple detection methods such as Size Exclusion Chromatography (SEC), Field Flow fractionation (FFF), Analytical Ultracentrifugation, and Hydrodynamic Chromatography. These techniques have provided powerful information about starch conformation that was useful for the design of this project**.**

Chapter 2 Page 42-52	**Influence of the range of molecular weight distribution of beer components on the intensity of palate fullness**

The objective outlined in this chapter is referred to the standardization of the AF4/ MALS/RI technique for the determination of the MWD in samples of commercial beers as well as experimental beers produced at a small scale in the laboratory according to different mashing conditions. Also this analytical tool was used for the determination of the MWD of different commercial maltodextrins and for maltodextrins added to samples of the same pilsner beer (spiking trial). This technological tool was combined with sensorial analysis of the beer samples to determine the influence of the MWD of the beer components on the palate fullness palate. The results showed that the MWD of commercial beers was closely associated with their palate fullness. It was also confirmed the influence of the concentration of maltodextrin on the beer palate fullness which was highly dependent on its MWD. Variations on technological parameters during mashing such as the initial temperature and the quality of the malt used also influenced significantly the palate fullness of the beer.

Chapter 3 Page 53-62	**Determination of the influence of starch sources and mashing procedures on the range of the molecular weight distribution of beer using field-flow fractionation**

This chapter describes the influence of distinct technological parameters such as the use of different mash mixtures (barley malt, barley malt +30% pre-cooked maize, barley malt + 30% nonmalted spelt) and distinct mashing procedures (infusion and decoction), with variations of the rest time and initial temperatures in infusion procedures, on the MWD of beers produced experimentally in the laboratory. The range of the MWD of the beer samples was determined using the AF4/ MALS/RI method as described in the previous chapter however separation conditions in AF4 were adjusted for the determination of MWD and rms in mash samples. For each of the beers produced under different mashing conditions, routine beer quality parameters such as the amount of total nitrogen and the apparent degree of fermentation (ADF) were also determined. The results showed that the use of infusion or decoction has no effect on the range of the MWD among the produced beers using similar raw materials and initial temperatures (45 and 55°C). However the range of MWD was significantly higher when using infusion at an initial temperature of 63°C, regardless of the raw material. The use of maize did not alter structural properties of the beer, while mash containing non-malting spelt caused significant elevation on the MWD accompanied by a significant lower of the ADF. Therefore the range of the MWD of the beers was influenced by the quality of the raw material and the initial mashing temperature whereas that ADF values were affected only by the type of starch source. Thus the range of the MWD is an important parameter for monitoring the quality of the production of beer under different mashing conditions.

Chapter 4 Page 63-69	**Analytical characterization of the hydrolysis of barley malt macromolecules during enzymatic degradation over time using AF4/MALS/RI**

This chapter refers to the use of the AF4 / MALS / RI technique developed for mash samples as described in the previous chapter in order to monitor the hydrolysis of starch that occurs throughout the mashing process in different isothermal mash/trials selecting mashing temperatures (65, 70, and 75°C) according to the activity of the alpha and beta amylase enzymes responsible for starch amylolysis during mashing. The results showed strong significant differences in the MWD and Particle size distributions (PSD) of the samples obtained at different times of the mashing process. These differences were closely associated with the activity of the enzymes at the selected temperatures. Thus, at mashing times over 30 min at a temperature of 65°C, when α- and β-amylase are both active, the decrease over time of the MWD and was significantly higher than at 70°C when mainly α-amylase is active. At 75°C, also the activity of α-amylase decreased and the MWD and PSD were significantly lower than at 70 or 65°C at any time of the procedure. The use of AF4/MALS/RI allowed determinations of the MWD and PSD of the samples at any point of the mashing procedure indicating its usefulness for monitoring starch degradation processes and its relation to the quality of the beer produced.

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REVIEW

An overview of separation methods in starch analysis: The importance of size exclusion chromatography and field flow fractionation

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Starch is the most important source of human food energy and also an important polymer for biofuels, processed food, and pharmaceutical industries. Precise determination of structural and functional properties of starch has been an outstanding factor for the improvement of dissolution and degradation processes, which are important procedures for commercial applications. Distinct separation techniques coupled with multiple detection methods such as Size Exclusion Chromatography (SEC), Field Flow fractionation (FFF), Analytical Ultracentrifugation, and Hydrodynamic Chromatography have provided powerful information about starch conformation. In the present review, we describe the usefulness and limitations of different separation methods particularly SEC and FFF, which are currently widely used.

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Keywords:
AP / AM / FFF / SEC / Starch analysis

1 Introduction

Starch is a polyglucan produced by plants, occurring in nature as water-insoluble granules, functioning as carbohydrate storage. It is the most important source of human food energy and an outstanding polymer for biofuels, processed food, and pharmaceutical industries [1]. It is mainly composed of two polymers: AM and AP, which are very different structurally, AM being linear and AP highly branched [2, 3]. Physical and chemical properties of starch such as viscosity, shear resistance, gelatinization, textures, solubility, tackiness, gel stability, cold swelling, and retrogradation are functions of their AM/AP ratio and other structural features [4, 5]. Also, the average MW and radius of gyration constitute important properties for commercial processing [4]. Starch samples contain a vast range of MWs distributions (MWD) and size distributions [4, 6] which may determine rheological and more general technological properties of the polymers. It must be considered that for polymers with branched chains, there is no unique relation between the size of the molecule and its molar mass in contrast to the situation of linear polymers of uniform geometry, where size and molar mass are

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Abbreviations: AF4, asymmetric flow-field flow fractionation; **AUC,** analytical ultracentrifugation; **BMIMCl,** 1-butyl-3-methylimidazolium chloride; **BMIMdca,** 1-butyl-3-methylimidazolium dicyanamide; D_t, translational diffusion coefficient; **DLS,** dynamic light scattering; **DMAc,** dimethilacetamide; **FACE,** fluorescence-assisted CE; **FFF,** field flow fractionation; **GFC,** gel filtration chromatography; **GPC,** gel permeation chromatography; **HDC,** hydrodynamic chromatography; **HPAEC,** high-performance anion-exchange chromatography; **HPSEC,** high-performance size-exclusion chromatography; **IUPAC,** International Union of Pure and Applied Chemistry; **MALLS,** multi angle laser light scattering; **MW,** molecular weight; **MWD,** MWs distributions; **NMMO,** *N*-methylmorpholine-*N*-oxide; **PSD,** particle size distributions; **QELS,** quasi-elastic light scattering; R_g, gyration radius; R_h, hydrodynamic radius; **SBEs,** starch-branching enzymes; **SdFFF,** sedimental field flow fractionation; **SEC,** size exclusion chromatography; **ThFFF,** thermal field flow fractionation; **UV,** ultraviolet; **Vh,** hydrodynamic volume uniquely related [4, 6]. A range of different molar masses can correspond to a same size, and any structural characterization must include some quantification of the branching, including connectivity [4, 6]. Measurement of this structural complexity in a way that can be useful for structure/property relations is still a major challenge.

Therefore, in the present review we describe different procedures involved in starch analysis, particularly the usefulness of different separation techniques such as size exclusion chromatography (SEC) and field flow fractionation (FFF) that have contributed to obtain reliable information about the structure of starch.

2 Starch structure

Starch granules are complex semi crystalline structures composed basically of two homopolymers of D-glucose: AM and AP which make up 98–99% of the dry weight of the granule, with the remainder comprising small amounts of lipids and minerals. Water content of native starch is about 10% [2, 7, 8]. AM is a linear, α-(1,4)-linked polymer (Fig. 1) whereas AP corresponds to a highly branched polymer formed by chains of α-(1,4)-linked glucose with α-(1,6) – branching links (Fig. 2) [2]. AM has a MW range of 10^5–10^6 g/mol and a degree of polymerization by number (DPn) of 324–4920 [7]. AP is much larger with a MW of about 10^8 g/mol and a DPn ranging from 9.600–15.900 [7].

Less than 0.5% of the glucoses in AM are in α-(1,6) linkages, resulting in a low DB [2, 3] leading to a tendency to form insoluble aggregates after dissolution [2]. AP has about 5% of its glucoses in α-(1,6) linkages, exhibiting a highly branched, tree like, structure and a complex molecule architecture that can vary substantially between different starches according to the position and length of the branches [2, 3]. The branches can be classified as: A-chains, which are unsubstituted, B chains, substituted by other chains and C chains corresponding to a single chain carrying the reducing glucose residue [2].

Starch granules vary in size (from 1 to 100 μm diameter) and shape (polygonal, spherical, lenticular) according to their botanical origin. They differ in the content of the AM and AP molecules. wx starches contain $<$15% AM, normal 20–35%, and high AM starches greater than about 40%. Also they vary greatly regarding structure and organization of AM and AP molecules [2, 7, 8]. Granules may occur individually or clustered as compound granules. In wheat, barley, rye, and triticale they occur in bimodal size distributions [2, 8].

The structure of the starch granule has been previously described [2, 3, 7, 8, 9, 10, 11, 12, 13]. Briefly, starch granules exhibit a hierarchical structure (Fig. 3) made up by alternating semi-crystalline and amorphous shells, named growth rings, ranging from 120 to 400 nm in thickness, which extend from the center of growth towards the surface of granules. It has been proposed that the semi-crystalline shells are formed by the association of several blocklets [8, 12] which are thought to be spherical structures (20–250 nm) formed by units of associated semi crystalline lamellae. The semi-crystalline structure in turn consists of repeat units of crystalline lamellae alternating with amorphous lamellae with a periodicity of approximately 9 nm [2, 8, 12]. The crystalline lamellae are assumed to be formed by clusters of AP short chains (12–20 glucose units) joined by longer chains (40 or more glucose units), which span two or three more clusters [3, 7, 9, 10, 12]. Adjacent short chains within the clusters

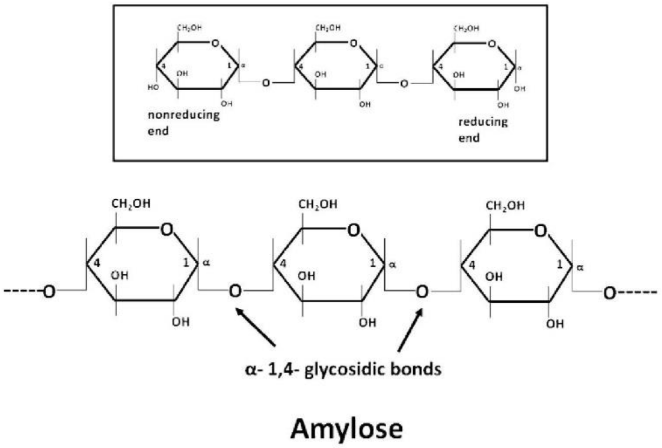


Figure 1. Schematic view of AM showing α-(1,4) glycosidic bonds and the reducing and non-reducing ends.

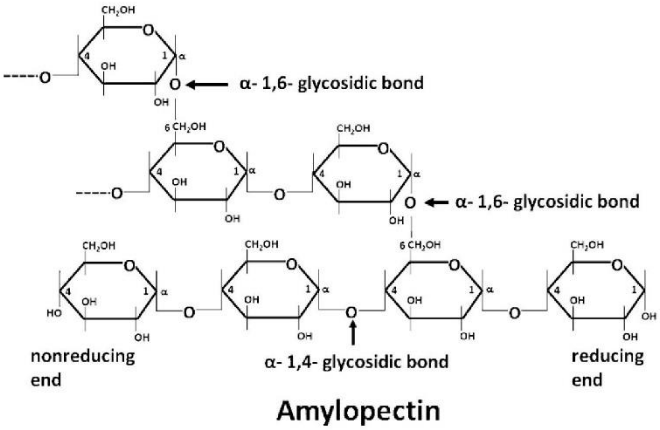


Figure 2. Schematic view of AP showing α-(1,4), α-(1,6) glycosidic bonds and the reducing and non-reducing ends.

form left handed helices that pack together in a tetragonal array leading to the crystalline structure of the lamellae. The branching points may be located within the amorphous lamellae [3, 7, 9, 12]. Because neighbor helices interpenetrate each other, the crystalline lamellae may form a more or less continuous super helical structure with a diameter of approximately 18 nm and a pitch of 10 nm [12]. A central cavity within the super helices would have a diameter of about 8 nm. Also, it has been proposed that the pitch of the helix originates from the clustering of branch points, and may be a characteristic of the botanical source [12]. The location of AM within the structure of the granule is still not well elucidated. AM may be located as individual chains within the amorphous zone of the growth rings [2, 3, 7, 8] although, it has been reported that AM can also be found randomly interspersed amongst the AP clusters in the semi crystalline lamellae [9, 11, 12]. Within the lamellae, AP can be arranged into either A- or B-crystalline forms, identified by characteristic X-ray diffraction (XRD) spectral patterns, being the packing of the helices in the A-type crystalline structure more compact with shorter chains than in the B-type, which exhibits a more open structure with a hydrated core and longer chains [2, 7, 8]. Cereal starches tend to have the A-type pattern, whereas the B type pattern is often found in tuber starches although both types may occur together. Legume, root, and some fruit and stem starches may yield an intermediate C-type pattern [7, 8, 12].

3 Starch dissolution processes

The rate of dissolution of a particular starch sample is an intrinsic physical property, which might correlate with

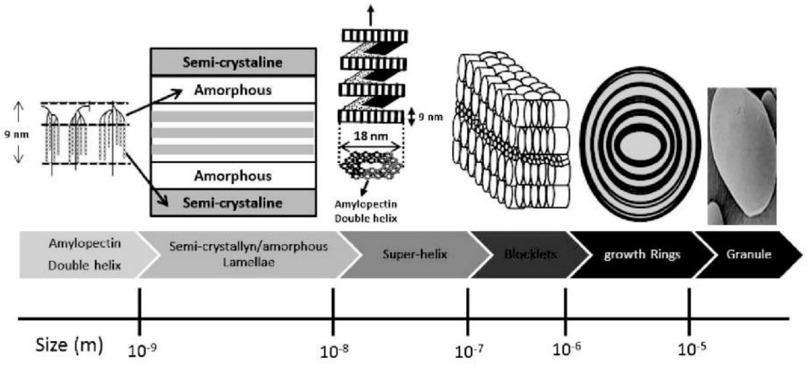


Figure 3. Schematic view of the hierarchical order of starch structure. Adapted from [7], [10] and [12]. The granule picture at the right of the scheme is a courtesy of the Institute of Brewing and Beverage Technology, TUM-Weihenstephan.

structural characteristics such as the arrangement of chains in the semi-crystalline starch granule providing important information about starch conformation [4, 14]. It is important to find procedures, which dissolve all of the starch in a sample without further degradation, avoiding the formation of aggregates or other entities resulting in incorrect apparent size distributions [4]. Starch is only partially soluble in water, at temperatures of 50–80°C and/or high pressure, although under these conditions starch is prone to debranch and dissolve into lower chain molecules affecting assessment of structural properties [15]. Furthermore, aqueous solvents, which solubilize starch, such as sodium hydroxide solutions, may also degrade it [14–17]. Dissolution issues have been overcome through the use of polar organic solvents with lithium salts. DMSO has been used for starch separation by SEC [18]. It decreases the handling time of each sample by eliminating sonication, filtration, and centrifugation steps required by other techniques such as the NaOH solubilization. Eluents composed of DMSO, Dimethilacetamide (DMAc) and lithium bromide has been successfully tested for starch separation using SEC [19]. In this approach, full dissolution in DMSO is followed by separation in a lower viscosity medium containing DMAc to limit shear degradation [19]. Using this eluent system, repeatability and reproducibility of the separation by SEC-RI, was very high, performing dissolution and separation at a relatively high temperature (80°C) [19]. Other studies showed that stearate esters of maltodextrin can be obtained in various degree of substitution (DS) when vinyl stearate or stearic acid was heated with maltodextrin in ionic liquid, 1-butyl-3-methylimidazolium dicyanamide (bmim[dca]) [20]. In this work, reaction temperature of 75°C gave the highest value of DS (0.64) and efficiency of 64%. Structure and DS of the resulting esters were confirmed by NMR, titration, SEC, and elemental analysis [20]. More recently, dissolution of starch in *N*-methylmorpholine-*N*-oxide (NMMO) was characterized by DSC. Exothermic transitions were observed for the three starches in both 78% and 70% NMMO whereas at 60% and 50% NMMO, the transition changed to an endotherm. Moreover, hot stage microscopy showed that starch granules dissolved at NMMO concentrations of 78% and 70%. In contrast, gelatinization behavior similar to that found when starch is dissolved in water was observed at concentrations of 60% and 50% NMMO [21]. Organic solvent systems may prevent the polysaccharide adsorption onto the stationary phases employed in techniques like SEC [4] nevertheless they can cause the deterioration of the filter membranes when using other separation technique like FFF. Thus, other approaches like dissolution by heat treatment at elevate pressure [22] should be more adequate when using FFF. In this case analysis of the samples on the same day at which they are prepared is recommended.

Starch dissolution can be monitoring using techniques such as polarized optical microscopy [23], DSC [24], static light scattering (SLS) [25], NMR [26] and dynamic light scattering (DLS) [26] whereas retrogradation, has been studied by rheology, XRD, thermal analysis and NMR [27]. Because DLS is sensitive to both: dissolved starch chains and aggregates, this technique should be more powerful to follow dissolution kinetics than NMR which is sensitive only to the dissolved starch chains but may be not sufficiently accurate to follow the kinetics of dissolution [26]. Besides, NMR has been improved using an in situ time-resolved NMR (consisting of recording sequential ^{1}H spectra of a sample in the spectrometer) that can detects quantitatively and specifically the dissolved species allowing directly follow of the kinetics of dissolution [26]. Polarized optical microscopy, which follows the disappearance of crystallinity [23] may be complementary to the time resolved ^{1}H NMR method. This approach can be applicable on wide time scales (from 10 min to several hours) and extended to other polysaccharide systems [26].

4 Degradation of starch

4.1 Enzymes involved in starch degradation

Enzymes involved in the breakdown of starch chains include four types: Amylases which hydrolyze α-(1,4)-glycosidic bonds; isoamylases which hydrolyze α-(1,6)-glycosidic bonds; glucanosyltransferases which transfer α-(1,4)-glycosidic bonds and branching enzymes transferring α-(1,4)- and α-(1,6)-glycosidic bonds [28]. The amylases are classified into endo-acting α-amylases (EC 3.2.1.1), exo-acting β-amylases (EC 3.2.1.2) and isoamylases or limit-dextrinases (EC 3.2.1.142) [28].The α-amylases attack the starch chain at glucose residues at (Fig. 4) in the interior part of the starch chain. Starch hydrolysis by α-amylase involves the interaction of an enzyme in solution with a solid substrate. Thus, the surface area accessible to the enzyme and the efficiency of the adsorption of the enzyme onto this surface are critical kinetic parameters [29]. The products of hydrolysis may vary according to the source of the enzyme. For example, the products of porcine pancreatic α-amylase are mainly maltose, maltotriose and maltotetraose [30]. The α-amylase from *Bacillus amyloliquefaciens* produces primarily maltotriose, maltohexose and maltoheptose [31] and that from *Aspergillus fumigatus* produces only glucose as the sole end degradation product [32]. The α-amylases can simultaneously solubilize both amorphous and crystalline regions of starch granules [33, 34] and there is no preferential hydrolysis of either AM or AP. The exo-β-amylases act on the non-reducing ends (Fig. 4) of starch polymer chains [28]. There are two general classes of β-amylases, those which

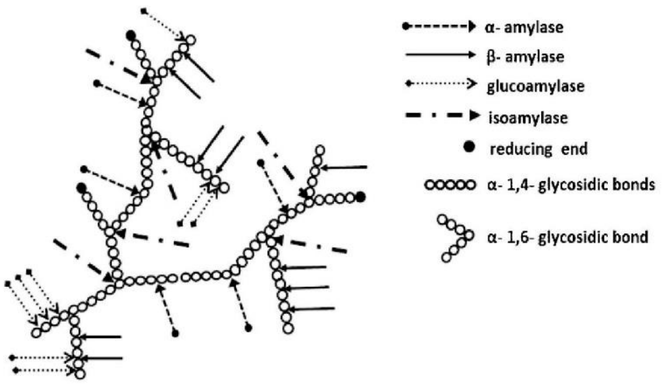


Figure 4. Schematic view of an AP molecule showing where the different types of enzymes attack. Courtesy of the Institute of Brewing and Beverage Technology, TUM-Weihenstephan.

are classically known as β-amylases and produce β-maltose and glucoamylases that produce β-D-glucose [28]. Maltose β-amylases are found in plants and have been isolated from sweet potatoes, soybeans, barley, and wheat [35]. They can also be produced by bacteria such as *Bacillus polimyxa*, *Bacillus cereus* and *Bacillus megaterium* [36]. Glucoamylases are exo-acting enzymes that release consecutive glucose units from the non-reducing ends of starch molecules (Fig. 4). They are mainly microbial enzymes, present in bacteria and fungi [37]. Fungal glucoamylases are biotechnologically important as they are used industrially in large amounts and have been extensively studied during the past 30 years. Prokaryotic glucoamylases are of biotechnological relevance for being generally thermophilic enzymes, active at elevated temperatures [37]. Isoamylases hydrolyze the branch linkages of starch (Fig. 4). They act by an endo-mechanism hydrolyzing the branch linkages located in the interior parts of the AP molecule to give linear maltodextrins without altering the α-configuration of the molecule [28]. The end products are maltotriose and malto oligosaccharides often with maltose and glucose [38]. They constitute an important source of enzyme for food industries being the *Pseudomonas* isoamylases the most studied [38].

Molecular approaches have contributed to the enhancement of the enzyme performance both in natural and synthetic environments, leading to the improvement of substrate specificity, activity, enantioselectivity, and thermal stability of different enzymes, providing processes of lower contamination, enhanced substrate, product solubility, and overall productivity [39] which may be important factors for successful starch degradation. Starch susceptibility to enzyme attack is influenced by the AM and AP content, crystalline structure, particle size, and the presence of enzyme inhibitors [40–44]. On the other hand, α-amylase affects the characteristics of starch granules. The enzyme can erode the outer surface of granule and also may digest channels leading to the granule center, which weakens granule integrity and leads consequently to its breakdown [45, 46]. Due to their structural properties, such as absence of pores on the granule surface, uniformity in granule size and variations in starch chain interactions within the amorphous and crystalline domains, legume starches make better substrates than cereal or tuber starches [42].

Functional properties of starch also influence digestibility [41, 43]. Gelatinization occurs when native starch is heated in the presence of sufficient moisture. The granules absorb water and swell and the crystalline organization is irreversibly disrupted [47]. Both heat and pressure during the preparation of starch suspensions alter the progression towards gelatinization increasing the availability of polysaccharide starch chains to digestive enzymes favoring hydrolysis [41, 47]. Retrogradation of starch may occur as the paste cool, forming insoluble crystalline aggregates, which may differ in form from the native granules [48]. Starch retrogradation, which depends on the time, temperature of sample storage and the AM/AP ratio [48], has also been correlated to rates of enzyme-catalyzed hydrolysis [49]. Both storage conditions and the fraction of AM, which affect re-crystallization [48] are correlated with the digestion rates of retrograded starch samples [41, 49].

4.2 Monitoring of starch degradation

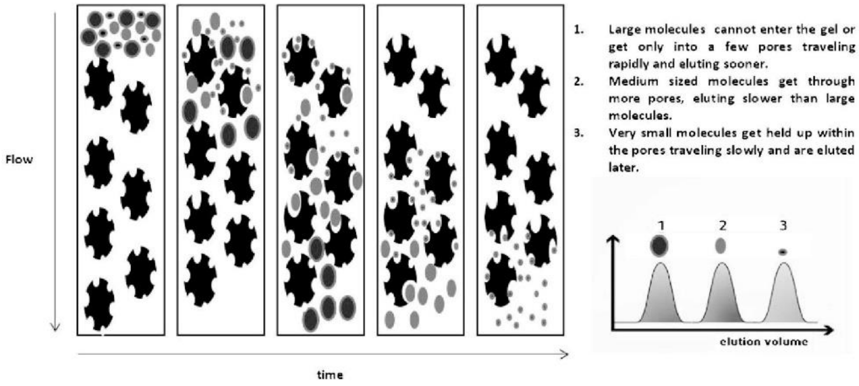
Digestion of starch can be monitoring by different techniques. Measuring the extent of the reducing capacity of the filtrate of aliquots, taken at intervals during the reaction, has demonstrated to be a suitable approach [50–52]. SEC methodology can be used for determining changes in the molecular size distributions of the carbohydrate substrate along the digestion process [41]. As explained below, SEC separates by size instead of by MW. This may be a disadvantage for monitoring starch digestion because AP is a branched polymer in which there is no unique relationship between hydrodynamic radius and MW, making difficult the determination of quantitative size distribution of AP in a sample without degradation [53–55]. However, SEC has been used to measure the time evolution of the distributions of molecular size and of branch length as starch is subjected to in vitro digestion, including studying the development of enzyme-RS [56]. The method was useful for the purpose of semi-quantitative comparisons between different starches undergoing the process of digestion, by observing the changes in their molecular structures, as an adjunct to detailed studies of the enzyme-resistant fraction [56]. Fluorescence-assisted CE (FACE) [57, 58] and high-performance anion-exchange chromatography (HPAEC) [59] have been used to obtain the distributions of oligosaccharide products, including the chain length distribution of the AP after treatment with a debranching enzyme [6]. Nevertheless these techniques are restricted to relatively low degrees of polymerization [53].

NMR methodology has also been developed to monitor progress of physical and chemical processes in starch suspensions [26, 60]. Introduction of ^{1}H NMR spectroscopy for the direct analysis of the reducing ends of oligosaccharides produced during hydrolysis has improved this approach enabling the accurate estimation of product formation during digestion of carbohydrates [60]. Recording the progress of a reaction using ^{1}H NMR spectroscopy is less time consuming than other techniques providing large data sets. However, NMR equipment is expensive making it unsuitable for industrial purposes. Also, most applications of NMR require deuteraded solvents, making the technique even more expensive [60]. Besides, other analytical methodologies such as asymmetric flow-FFF (AF4) may be also suitable for monitoring the process of starch digestion [61]

5 Separation methods

5.1 Size exclusion chromatography (SEC)

SEC appeared in the late 1950s. It was first named gel permeation chromatography (GPC) or gel filtration chromatography (GFC) [4, 62, 63]. Because the separation is based on size exclusion, IUPAC recommends the term SEC. It plays an important role in the determination of molar masses and molar mass distributions of linear homopolymers, assessment of molecular parameters of branched polymers, assignment of sizes of isolated macromolecules in solution, as well as of the magnitude of their possible aggregation and association [62]. The principle is based on the sorting of molecules according to their sizes in solution and has been described elsewhere [4, 14, 53, 54, 62, 63]. Briefly, the sample solution is injected into the column, which is filled with rigid, porous, materials, and is carried by the solvent through the packed column [54, 62, 63]. The sizes of molecules are determined by the pore sizes of the material of the packing column within where the separation occurs (Fig. 5). The pore size of the column packing has to match the sizes of macromolecules and the mobile phase must be able to dissolve it [63]. It is important to prevent polymer-packing interactions in the routine SEC measurements. If the column packing contains active sites that interact with separated macromolecules, the resulting retention volumes can be altered [62, 63]. The primary purpose of SEC is to provide MW distribution information about a particular polymeric material in a short period of time [62, 63]. The



1. Large molecules cannot enter the gel or get only into a few pores traveling rapidly and eluting sooner.
2. Medium sized molecules get through more pores, eluting slower than large molecules.
3. Very small molecules get held up within the pores traveling slowly and are eluted later.

Figure 5. Principle of the separation of molecules by SEC. The graphic at the right of the picture showing elution volume according to the size of the particles is a courtesy of Polymer standards Service GmbH (Mainz, Germany).

parameter by which separation occurs is termed hydrodynamic volume (V_h), which in SEC is proportional to the product of the weight-average intrinsic viscosity and number-average molar mass [62, 63]. The values can be determined with standard calibration by the use of standards with well-known MW and intrinsic viscosities [54, 63]. The V_h, and corresponding hydrodynamic radius (R_h), is related to the radius of gyration (R_g) [53]. R_g describes the average distance of the polymer segments from the center of gravity of the polymer, whereas R_h is a measure of the hydrodynamically effective sphere radius of the polymer [64]. The relationship between these two quantities depends on the nature and structure of the polymer. The ratio of these two parameters gives useful information on the solution conformation of the macromolecule of interest [53, 54, 62, 63, 64].

For optimal use, SEC must be coupled with different detection methods such as RI, UV absorption, low angle light scattering (LALS), multi angle laser light scattering (MALLS), viscometry, and DLS. RI and UV absorption detectors are sensitive to the concentration of the sample [65–67] and are most commonly used. The advantage of an RI detector is its ability to detect almost any polymer. It can be used for Universal calibration methods or in combination with light scattering [65, 67]. UV detector measures absorption of a solute sample and can be applied to measure concentration of proteins and polymers. The instrument measures absorption at a specific wavelength. The analyzed molecules need to contain a chromophor-like double bond, such as aromatic rings, carbonyl groups, etc., but it cannot be used for polymers, which not contain such functionality [67]. LALS, MALLS are molar mass sensitive detectors, which are very useful in SEC [66, 67]. Nevertheless, the information, which can be obtained from each detector, is different. From both MALLS and LALS the absolute molar mass distribution and Mw can be determined directly [67]. However, from LALS (measuring the scattering intensity at just one angle), no information is obtained on polymer conformation and radius whereas with MALLS it can be also obtain the R_g [66, 67]. In DLS or quasi-elastic light scattering (QELS) time dependent fluctuation in the scattered light, measured by a fast photon counter, is directly related to the rate of diffusion of a molecule through the solvent [66]. The technique allows the determination of the R_h and the translational diffusion coefficient (D_t) of the sample [66]. On the other hand, the use of online viscometry in SEC in conjunction with a universal calibration curve leads to the determination of the number-average MW [65]. Because different and complementary information can be obtained from distinct detectors, most of the current approaches use combination of two or more detector systems. For example, studies of AM and potato starches of different origins were carried out by DLS and HPSEC-MALLS methods [64]. In this work, the use of SEC-MALLS yielded precise values of MWD, Mw and R_g for isolated and synthesized AM as well as for potato starches, whistle DLS allowed the determination of R_h. The results from the combination of SEC-MALLS and DLS leaded to the determination of the structure-sensitive parameter: R_g/R_h. The magnitude of this parameter depends on the architecture of the polymer thus giving information about the polydispersity of the polymer chains [64].

As mentioned above, SEC has been useful for the analysis of the distributions of molecular size and of branch length during the process of starch digestion [56]. Among many other applications, SEC analysis has been a tool for the mechanistic investigation of starch-branching enzymes (SBEs) [6], for studying the effect of storage on the quality of maize starches [68] and the analysis of AM contents of enzymatically modified corn starches [69]. Nevertheless, shear degradation of native starch, particularly of the AP component has been extensively reported [4, 14, 53, 54] using SEC even in appropriated solvent systems [54]. Therefore, the applicability of conventional SEC on the analysis of the size distribution of starch would be limited to smaller and less branched molecules [53].

5.2 Field flow fractionation

FFF is a versatile technique that may provide fast, gentle and high-resolution separations of any particulate matter from 1 nm up to 100 μm in a liquid medium. It was patented in 1966 by Prof. Calvin Giddings and collaborators [70]. They also developed Thermal Field-Flow Fractionation [71], Sedimentation Field-Flow Fractionation [72] Flow Field-Flow Fractionation [73], and Split Flow Thin Cell Fractionation [74]. All these versions utilize the same basic separation principle, but employ different force fields. The FFF principle does not rely on interaction of the molecule with a stationary phase but with an externally generated field, which is applied perpendicularly to the direction of the mobile phase flow [75]. FFF allows separation of nearly any particle in the correct size range like metal particles, high MW polysaccharides, liposomes, viruses, functional proteins like enzymes, living cells, non-covalent aggregates, etc. The mechanism of the separation has been previously described [76, 77]. In this technique, a laminar flow field is applied perpendicularly to the parabolic flow to drive the molecules to separate into different laminar flows due to differences in their diffusion coefficient. This results in different retention times (Fig. 6). In the normal elution mode, retention times are shorter for lower molar mass/size molecules. Next to the diffusion coefficient also the charge and the shape can influence the retention time [76].

The second flow stream (cross flow) controls the separation force makes the flow FFF (F4) possible [74].

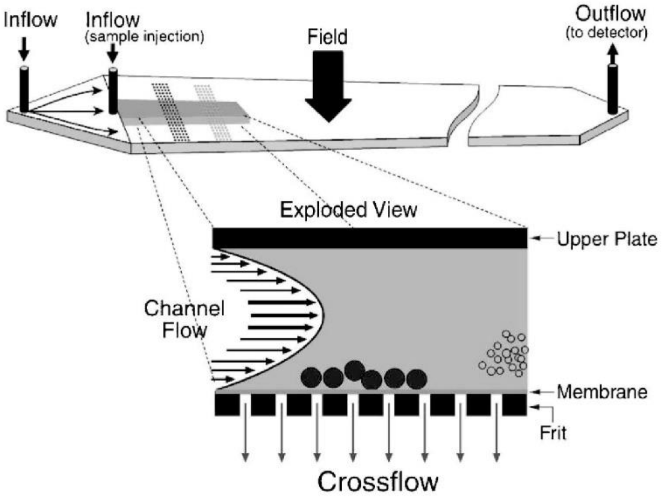


Figure 6. Principle of the separation of biopolymers by asymetrical flow FFF (AF4). Reproduced with permission from Wyatt Technology Europe.

Currently, two techniques are commonly used in the market as symmetrical F4 (SF4) and asymmetrical F4 (AF4) [72, 77]. In AF4, an impermeable upper plate constitutes the upper wall of the channel. The bottom channel plate is permeable and made of a porous frit material together with an ultrafiltration membrane. The cross flow is generated directly inside the channel, where the main flow coming from the inlet is divided to generate a cross flow through a semi-permeable membrane that is located on the bottom wall, while the rest of the longitudinal flow stream is directed to the detector system (Fig. 6). The membrane pore size is selected in such a way that only the solvent can pass through while the sample particles are retained. The availability of different size pore membranes allows a better selectivity for the determination of a particular MW range [76].

In AF4, the separation method contains two parts. The first part is called focusing. During focus, the sample is injected into the cannel and the particles oriented in a focus zone achieving equilibrium after injection flow is switched off. Smaller particles with a higher diffusion coefficient located in average higher as bigger particles with lower diffusion coefficient [76, 77]. AF4 separates molecules according to their differences in translational diffusion coefficient (D_t), which can be converted to R_h assuming a spherical geometry and applying appropriated equations [75, 76]. The applicability of AF4 has been demonstrated in the characterization of molecules ranging from solutes with inherent molar masses of approximately 10^3 up to about 10^9 g/mol, and of particles with dimensions of <2 nm up to about 50 μm. The lower size limit is determined by the MW cut-off of the membranes (approximately 10^3 g/mol), whereas the upper size limit is assessed by a threshold of about 20% of the channel thickness [76, 77].

In ThFFF, a temperature gradient is used to drive polymer components by thermal diffusion [78]. The movement of components down the channel toward the detector is determined by their mean steady-state position in the velocity profile. In ThFFF, the channel is open without stationary phase [78] providing excellent separation reproducibility, giving several advantages over SEC. For example, retention volume, limited to one column volume in SEC, is theoretically unlimited in ThFFF [79]. On the other hand, by programming the temperature gradient, polymer mixtures containing components with a wide range of MW can be handled in a single run [79]. Combining ThFFF with SEC [80] as well as with hydrodynamic chromatography (HDC) [81] has shown to be successful approaches for polymer characterization.

In SdFFF, also called centrifugal- or multigravitational-FFF, the external field applied is a centrifugation field, generated by the rotation of the separation channel in a complex device [82, 83]. In many studies, SdFFF have been successfully used to rapidly and easily analyze the particle size distribution of starch granules [84–86]. There has been much interest in combining information provided both by SdFFF (retention data) and flow cytometry (particle size distribution), in terms of size/density properties of different cell lines or starch [87, 88]. Nevertheless, to avoid carrier liquid leakages, the SdFFF apparatus requires sealing parts spinning at high speed, which involves some technical complexity increasing the cost of the device. Application of Earth's gravity should make Gravitional FFF (GrFFF) the simplest FFF version from a technical point of view. Nevertheless it is still a prototype technology with limited applications.

FFF coupled with multiple detection methods is a powerful tool for obtaining data of hydrodynamic radius, radius of gyration, molar mass, polymer conformation, and branching degree. It can be used to determine all properties important for polysaccharides with complex structures. UV–Vis detector is used mainly for protein and peptides analysis [89, 90]. The RI detector is the most widely used playing an important role in FFF applications, regardless its sensitivity and stability [90]. Combination of the separation capacity of FFF with the particle sizing power of MALLS created an approach with a great resolution that constitutes the basis of today's modern FFF-Light Scattering technology [91].

The technique has been successfully used in starch analysis. For example, AF4-MALLS-RI has been used to determine the nature of AP branches from native starch [5] demonstrating that AF4 enabled a good fractionation for AP leading to detailed macromolecular characteristics. It was shown that AP M_w, R_g, and the hydrodynamic coefficient v_G (the slope of the log–log plot of $R_g\,i$ versus $M_w\,i$) were within the ranges of 1.5–3.18 × 10^8 g/mol, 163–229 nm, 0.37–0.49, respectively [5]. It was reported that the data achieved for hyper-branched polymers followed an ABC theory model [5]. The relationship between R_g and the molar mass over the size distributions provided the information about the density and branching of the different samples. It was found that wx maize starch have the highest DB, followed by normal maize AP, cassava AP, wx rice starch, and smooth pea AP, wx barley starch, and finally AM free potato starch [5]. AF4-MALLS-RI was also used to study molecular size characteristics from AM and AP of different types of starch (maize, wheat, potato tapioca, pea and rice) [22]. In this survey, large differences on the amounts of AM and AP as well as on the molar mass and hydrodynamic diameters were found according to the plant source [22]. It was observed that for all the samples analyzed, the approach allowed the identification of two well-defined size populations representing mainly AM and mainly AP and an overlapping region in which AM and AP were possibly co-eluted in a range where they have similar hydrodynamic diameters [22]. Most of the first population had molar masses below 10^6 g/mol, and most of the second size population had molar masses above 10^7 g/mol [22]. Co-elution of AM and AP has also been reported by other authors [92]. In this work, using AF4-MALLS-RI, no separation of AM and AP from native sago starch was observed [92]. In this case, the effect was attributed to an overlapping of the diffusion coefficients of both AP and AM fractions [22, 92]. AF4-MALLS-RI has been successfully used for study the changes in the MW and the size distribution of corn starch during carboxymethylation [93]. It was shown that due to molecular degradation by alkaline treatment, the weight-average MW of the starch sample was reduced down to about 4.4 × 10^5 from about 7.2 × 10^6 g/mol. Among many other applications, the technique has been applied for the study of the size/conformation relationship in wx barley starch [94] and for monitoring the changes in the MW and the size distribution of starch during derivatization [95]. A recent study have reported the use of AF4-MALLS-RI for the characterization of starch from "Mapuey" tubers that are staple food for indigenous peoples from the Venezuelan Caribbean coast and Amazon regions [96] allowing these researchers to discover a new natural AM-free starch in the neglected yam "Mapuey" that could represent some potential for local food industry. More recently, AF4-RI-MALLS-QELS combined with HDC-SEC-RI-MALLS–QELS, facilitated the study of the structural properties of native starches displaying different AM/AP ratios [97]. The procedure, based upon DMSO pretreatment, generated a representative injected sample without altering the initial degree of polymerization. The AP weight-average molar masses and R_g were around 1.0 × 10^8–4.8 × 10^8 g/mol and 110–267 nm, respectively. For each starch sample, the R_h distributions and the molar mass distributions obtained from the two-fractionation systems coupled with RI, MALLS and QELS were analyzed. The size determination scales were extended by means of R_h calibration curves. HDC-SEC and AF4 data could be matched. However, results showed that AF4 enabled a better separation of AP and therefore an enhanced structural characterization of the starches. The main advantages of this approach were the direct determination of distributions as a function of both molar mass and size, while taking account of sample heterogeneity, as well as the possibility to compare the results obtained using the different techniques through the direct application of R_h distributions [97]. Other variants of the FFF technique have been suitable for carbohydrate analysis. For example, High temperature asymmetric flow-FFF (HTAF4)-MALLS-RI and viscometry has been used for the characterization of high MW polyethylenes that cannot be accurately resolved by other methods [98]. Application of ThFFF-MALLS-RI was used to study polydisperse polymer mixtures containing ultra high MW polymers and microgels in organic solvents [99]. In addition, SdFFF has been applied to study starch amylolysis on monomodal (native rice starch) [100] and bimodal (native wheat starch) [101] populations. Also, a recent application of GFFF in the study of the distribution of starch granules during amylolysis has being reported [102].

5.3 Alternative separation techniques

HDC and Analytical ultracentrifugation (AUC) has also been used in starch analysis. HDC has been developed in micro-fabricated devices [103]. Currently, is being considering as an attractive alternative to the classical separation methods (SEC or FFF) for starch analysis [104]. The main advantages would be fast analysis, high separation efficiency, negligible solvent consumption, and easy temperature control [103, 104]. However, the device still needs further improvement. AUC is a classical method described elsewhere [105]. Because it relies on the principal property of mass and the fundamental laws of gravitation does not require standards for comparison. For example, it has been successfully used to characterize and quantify the molecular composition of starches from different sources that had been physically damaged to different extents [106].

6 Limitations and perspectives of FFF and SEC

Regardless of the purpose, separation of starch into its components is a complex process and currently there is not a solely, best, technique to satisfy all kind of demands. Therefore, the choice of an appropriated approach will depend of the objectives of a particular project. For example, assessment of the R_g using SEC-MALLS would be limited for small particles ($<$10 nm) because at this size range no angular dependence of the scattered light can be observed. On the other hand, QELS can give the hydrodynamic radius (R_h) of samples including smaller particles of about 1 nm. Therefore, coupling QELS with SEC-MALLS would be a complementary approach allowing precise determination of R_h for smaller particles (1–20 nm) and R_g for particles $>$20 nm in which the angular dependence of the scattered light is significant. The disadvantage of online-QELS lies in the very low concentration of sample inside the MALLS-flow cell due to dilution of the sample during separation. Significantly more of the injected sample would be required for online-QELS than MALLS. This would be a disadvantage when using FFF because the system may over load as greater quantities of injected sample are needed, which would not occur easily when using SEC. Therefore coupling QELS with SEC would be more appropriated than coupling this device to FFF, particularly for separation of small particles (1–20 nm). Besides, according to the principle of SEC the observed tendency is that lower particles, which elute later, would separate more efficiently by this technique. In contrast, for the separation of greater molecules (20–600 nm) the use of FFF coupled with MALLS allows determination of R_g without any limitation. In addition, the availability of separation membranes with different pore sizes (1–30 kDa) allows a better selectivity for the determination of MWD and PSD using FFF. On the other hand, FFF relies on the diffusion of molecules and particles against a flow field into parabolic flow inside a channel. The lack of a stationary phase in the channel removes the effect of shear force present in SEC and also yields better separations of poly disperse systems like polysaccharides, complex polymers and colloidal suspensions among others. In addition FFF provides the advantage to avoid clogging the column; membranes can be easily replaced lowering the overall costs of material replacing.

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ORIGINAL PAPER

Influence of the range of molecular weight distribution of beer components on the intensity of palate fullness

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Abstract The effect of the molecular weight distribution (MWD) of the beer components on the intensity of palate fullness was studied. The range of MWD for different types of maltodextrin and for nine commercial pilsner beers was determined using AF4/MALLS/RI. Sensory analysis (DIN 10952, DIN ISO 4120 and DIN 10963/ISO 8587) was carried out by a trained Deutsche Landwirtschafts-Gesellschaft, German Agriculture Society (DLG) tasting panel. The intensity of the palate fullness of a spiking trial (beer + maltodextrin) and the threshold concentration of the maltodextrins in beer was determined. The association between the ranges of MWD and the intensity of the palate fullness of the commercial pilsner beers was studied. AF4/MALLS/RI and sensory analysis were used to study the effect of variations of the brewing process on the range of MWD and the palate fullness of beer. The intensity of the palate fullness differed significantly ($p < 0.0001$) within commercial pilsner beers. Strong associations were found between the range of MWD of the commercial beers and intensity of the palate fullness ($p < 0.05$). The range of MWD of the commercial pilsner beers (3–13 kDa) corresponded to those found for maltodextrins with intermediated range of MWD (3.4–22.3 kDa). The threshold concentration was higher ($p < 0.0001$) for those maltodextrins with lower range of MWD (2.7–8.9 kDa). Beers produced with malted barley with Kolbach Index of 36 % exhibited a higher range of MWD (2.9–13 kDa) compared to those with Kolbach Index of 41 % (1.7–11.6 kDa). Slight differences in the palate fullness were perceived according to variations on the initial temperature of the mashing process among those beers produced using Kolbach Index of 36 %, whereas a great difference ($p < 0.0001$) was perceived using Kolbach Index of 41 %. The intensity of the palate fullness of the pilsner beer was influenced by the range of MWD of the beer components which would vary according to differences in the mashing process and of the quality of the malted barley.

Keywords Beer · Palate fullness · Molecular weight distribution · Maltodextrins · Mouthfeel · MALLS

Introduction

Beer is a fermented beverage mainly composed by water, ethanol, non-fermentable carbohydrates and a small quantity of proteins [1–5]. The proportion of these molecules determines the flavor and textural attributes defined as the mouthfeel of the beer [5, 6]. Malted barley which has an appropriate level of protein, low level of lipids and generates high levels of amylolytic enzyme activity during germination, is the most used source of carbohydrates (mainly soluble starch, arabinoxylan and β-glucan) for brewing processes [1, 5, 7]. Starch is a complex semi-crystalline structure composed basically of two homopolymers of D-glucose: amylose and amylopectin. Amylose is a linear, α-(1, 4)-linked polymer, whereas amylopectin corresponds to a highly branched polymer formed by chains of α-(1, 4)-linked glucose with α-(1, 6)-branching links [8]. The access of amylases to the granule which is essential for starch degradation [8] is determined by the grade of dissolution of the cellular wall of the starch granule [8] which depends on the activity of some

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proteases and β-glucanases also activated during the malting process [3, 9, 10]. Chemical degradation of the malt can be measured using the Kolbach Index which has been defined as the amount of soluble nitrogen (or protein) in a grain related to the total amount of nitrogen (or protein) [11]. Thus, malt with a Kolbach Index between 36 and 42 % is considered as highly modified and suitable for mashing [3, 9].

During mashing, the malt amylolytic enzymes break down the starch into fermentable sugars [9, 10]. Both α- and β-amylase can hydrolyze α-(1, 4) bonds. α-amylase hydrolyzes intact starch granule and degrades amylose to oligosaccharides, maltose and glucose [8]. However, amylopectin gives rise to small branched dextrins which cannot be further broken down during mashing [8, 10]. Thus, carbohydrates in beer include small residual fermentable sugars and a variable amount of higher dextrins (20–30 g/L in a pilsner beer), most of which contain two or more branches [1–5]. Dextrins are branched polysaccharides including poly-disperse species [12]. The most known are maltodextrins [13, 14] which are soluble dextrins with a higher reducing power than starch. They are usually characterized according to their dextrose equivalent (DE) which has been defined as the amount of reduced sugar expressed as glucose as a percentage of the dry substance weight. For example, maltose has a DE of 50. Maltodextrins are known to have a dextrose equivalent of less than 20 [14]. According to their DE value, maltodextrins have different physicochemical properties like solubility, freezing temperature and viscosity among others [13, 14]. However, maltodextrins with similar DE value may have very different properties depending on the hydrolysis procedure, botanical source of starch and amylose/amylopectin ratio [14].

The mouthfeel of the beer comprises three key attributes which are carbonation, palate fullness and aftertaste [15–17]. Carbonation is often the first attribute perceived in the mouth. It is felt as a particular sting or tingle that is linked to the amount of carbon dioxide in the beer [15–17]. Palate fullness refers to the weight and flow resistance of a beer while it is being consumed [15]. The physical properties associated with the palate fullness are density and viscosity [15]. The final attribute of mouthfeel is the aftertaste which is associated with the lasting sensations recognized in the mouth such as stickiness, astringency, dryness and bitterness [15–17]. The aftertaste in beer is mainly determined by the bitter that comes from the hops [17]. Beer components influencing the perception of mouthfeel, mainly dextrins [18, 19], and small amount of proteins [15] can be considered to originate from its raw materials and the variation of the mashing process [20]. The characterization of the structural properties of these components would play an important role in the development of new technologies for the optimization of brewing processes in the industry. In this work, we evaluated the influence of the molecular weight distribution of beer components on the intensity of the palate fullness of the beer.

Materials and methods

Molecular weight distribution of maltodextrins and beer samples

The range of the molecular weight distribution (MWD) of maltodextrin in a spiking trial (beer + maltodextrin) as well as of different commercial pilsner beer and experimental beers produced in the laboratory was determined. To corroborate the accuracy of the methodology by asymmetrical flow field-flow fractionation (AF4) (Wyatt Technology, Germany) for the determination of the range of the MWD of different commercial maltodextrins, the range of the MWD of the maltodextrins alone (dissolved in water) was first determined. The procedure was carried out using 5 different types of commercial maltodextrins (Lehmann & Voss & Co, Hamburg, Germany), identified as M1, M2, M3, M4 and M5. For sample preparation, the corresponding amount of maltodextrin (10 g) was weighted and dissolved in 500 mL of water to reach a concentration of 20 g/L. Carbon dioxide was removed through a filtration process. For beer-maltodextrin samples, the same procedure was carried out using the same maltodextrins (M1, M2, M3, M4 and M5), and a concentration of 20 g/L was used for analysis. Commercial beer samples as well as experimental beers were filtered before further analysis to determine the range of MWD. All the samples (maltodextrin + water, maltodextrin + beer, commercial beers and experimental beers) were analyzed using AF4. The samples were measured three times. Weight average molar mass (Mw) and the range of molecular weight distribution (MWD) were determined by multiangle laser light scattering (MALLS) (Dawn, Heleos II, Wyatt Technology, Germany) and by refractive index (RI) as quantitative detector (Agilent Series 1200 G1362A, Agilent Technologies, Germany) [8, 21]. 100 μL of each sample was injected to the channel. For the separation process, the inserted spacer had a height of 490 μm, a width of 21.5 mm at the widest position approximately where the sample was first focused (500 seg) in the long channel (240 mm) before eluted to the detectors. Measurement was carried out at 25 °C. Carrier eluent was composed of 50 mM $NaNO_3$ and 200 ppm NaN_3 dissolved in Millipore water. Separation was conducted with a 1 kDa membrane (Polyethersulfon, Wyatt Technology, Germany). Elution flow was 1 mL min^{-1} and cross flow was decreased from 4 to 0.1 mL min^{-1} during 30 min. Then cross flow was constant for 20 min at 0.1 mL min^{-1} and finally reduced to zero. The intensity of scattered light was measured simultaneously at 18 different

Table 1 Molecular weight of standard solutions

Standard solution	Volumen (ml)	Manufacturer Molar mass (kDa)	Obtained Molar mass (kDa)
Pulullan 6 kDa	15,1	5,9	6,46
Pulullan 10 kDa	16,9	9,6	9,83
Pulullan 21 kDa	21,0	21,1	22,61
Pulullan 47 kDa	27,5	47,1	49,11
Pulullan 107 kDa	35	107	121,6
Pulullan 200 kDa	38	200	196,5
Pulullan 708 kDa	43,3	708	690
BSA	17,2	66,5	67,3

scattering angles ranging from 10° to 160°. A dn/dc of 0.146 mL g^{-1} was used to determine the concentration of polysaccharides in aqueous solvents by the RI detector. Baselines of RI detector were subtracted by baselines from blank runs since RI baselines are influenced by different salt and pressure conditions during a cross flow gradient in AF4. Before analysis of the samples, the accuracy of the calibration of the analytics (AF4/MALLS/RI) was verified. Pullulan standards (PSS GmbH, Mainz, Germany) of known molar mass (6–708 kDa; 1 g/L) as well as bovine serum albumin (BSA) (Sigma Aldrich), (66.5 kDa; 1 g/L) were measured. The results were compared with those provided by the manufactures. Table 1 shows that the values of MWD obtained by the AF4/MALLS/RI confirmed those reported by the manufacturer. The MWD expressed as the cumulative weight fraction as a function of the molar mass was obtained using the ASTRA V 6.0.3.16 software (Wyatt Technology, Germany).

Sensory analysis

Sensory analysis was carried out by the team of the chair of Brewing and Beverage Technology, Weihenstephan. Fifteen individuals certified as beer tasters by the Deutsche Landwirtschafts-Gesellschaft, German Agriculture Society (DLG) participated in this sensory evaluation. Previous to this work, they have been trained to acquire the ability to evaluate the mouthfeel of several types of beers [22]. The intensity of the palate fullness for each maltodextrin-beer sample, as well as the threshold concentration of the samples was determined. Also, the intensity of the palate fullness of different commercial and experimental pilsner beers was evaluated. The trials were conducted as follows:

Spiking trial

Preliminary trials To establish the adequate conditions for the spiking trial, a preliminary tasting was carried out. A concentration of 40 g/L of 5 different types of commercial maltodextrins identified as M1, M2, M3, M4 and M5 was added to different samples of the same pilsner beer. A total of 6 samples were presented to the tasting panel: A control commercial pilsner beer (without addition of maltodextrin) and 5 mixed stock model solutions (maltodextrin + beer). The maltodextrins were selected according their range of molecular weight distribution. For sample preparation, the corresponding amount of maltodextrin was weighted (40 g) and then dissolved in 500 ml of beer. With the aim of establishing adequate conditions for sensory analysis such as temperature and beer sparkling (CO_2 content), approximately 20 min before the tasting was held, 500 ml mixtures of maltodextrins-beer were mixed with 500 ml of fresh beer at 2 °C. The resulting samples were presented among the tasting panel (100 mL for each taste). All tests were performed at around 15 °C. Samples were evaluated according to their intensity of palate fullness. A scale from 0 (no sense) to 7.0 (extremely strong) was used according to sensory analytical procedures (DIN 10952) described by Gastl et al. [23].

Intensity of the palate fullness according to the concentration of beer-maltodextrins samples Different concentrations (2.5, 5.0, 10.0, 20.0, 40.0 and 80.0 g/L) of distinct maltodextrins (M1, M2, M3, M4 and M5) were independently added to samples of the same pilsner beer. All samples were prepared as explained above. Thus, six samples corresponding to five different stocks of maltodextrin-beer, at the same concentration and also a standard control pilsner beer (without maltodextrin), were randomly presented to the panel. For each maltodextrin concentration, three different tasting sessions were carried out. Samples were evaluated according to their intensity of palate fullness using the procedures described above.

Threshold taste of maltodextrins in the spiking trial The DIN ISO 4120 sensory analysis triangle test [23] was used to evaluate the threshold taste with respect to the standard pilsner beer, corresponding to each maltodextrin-beer sample. For this tasting, 3 coded samples for each concentration (1.25, 2.5, 5.0, 10.0, 20.0, 40.0 g/L) of maltodextrin were submitted, two of which were identical (i.e., 6 sets of 3 samples each), and these were arranged in ascending concentration. For this procedure, three samples were presented to the panel, and the tasters identified which of the three samples was different (odd sample). The maltodextrin taste threshold was defined as the arithmetic mean of the group threshold values at which the maltodextrins were detected unambiguously.

Palate fullness of different commercial beers

For determining the influence of the range of the MWD of pilsner beer on the intensity of the palate fullness, nine

Table 2 Alcohol and Wort extract content of the different commercial pilsner beers

Beer	Analysis 1		Analysis 2	
	p (g/100 ml)	Alcohol (% vol)	*p* (g/100 ml)	Alcohol (% vol)
1	11,47	4,59	11,51	4,63
2	11,55	4,57	11,56	4,59
3	11,52	4,64	11,57	4,69
4	11,61	4,74	11,64	4,76
5	11,48	4,65	11,53	4,67
6	11,65	4,68	11,69	4,73
7	11,57	4,62	11,71	4,75
8	11,68	4,81	11,63	4,78
9	11,73	4,71	11,76	4,72

different commercial pilsner beers, similar in alcohol and extract content (Table 2), were chosen from the market. The samples from each trademark belonged to the same production batch. Sensory analysis was carried out using a ranking test according to DIN 10963/ISO 8587 [24]. Beer samples were tasted at 15 °C. The beers were each time randomly presented for tasting, and the panel had to arrange and label the samples in an ascending order according to the intensity of the palate fullness. The procedure was carried out ten times.

Modification of technological parameters of the brewing process and evaluation of the MWD and the palate fullness of experimental beers

An amount of experimental beers were produced in the laboratory at a scale of 10 L. Different initial temperatures of the mashing process were tested in a range of 45, 55 and 63 °C, respectively. Figure 1 shows the evolution of the mashing procedures. All further phases of the mashing process (boiling wort, wort filtration, fermentation, beer filtration and beer filling storage) were performed identical, for all trials, according to standard procedures [25–27]. Briefly, boiling wort was performed during 80 min (98–99 °C). Primary fermentation was carried out at a temperature of 12 °C during 6 days. Secondary fermentation was carried out at a temperature of 15 °C at a pressure of 1 bar during 3 days. Lagering was performed during 3 weeks at a temperature of 0 °C. The obtained beers were stored at 2 °C. In order to obtain a good reproducibility of the results, four experiments were carried out for each temperature. The final wort concentration for each beer was of 11, 5 % W/V. For this purpose, 1.5 kg of malt for 10 L of wort was required. Common malt was made at the chair of Brewing and Beverage Technology, Weihenstephan, according to standard MEBAK procedures [25]. Malts with different Kolbach Index: 41 and 36 % (Mathe, Saaten union, Germany, produced with different steeping degree) were used [25]. All the experiments were carried out using infusion mashing process. Bitterness was similar for all trials (same quantity of hops during wort bowling) resulting in 20 IBU for each type of final beer. The range of the MWD for each resulting beer was determined using AF4/MALLS/RI as explained above. Sensory analysis was performed using a scale from 0 (no sense) to 7.0 (extremely strong) according to DIN 10952, described by Gastl et al. [23]. In this analysis, 10 tasting sessions were carried out. In each session, six beers were tasted (two for each temperature); therefore, the intensity of the palate fullness for each beer was evaluated five times.

Statistical analysis

Statistical analysis was carried out using GraphPad InStat version 3.05, (Graph Pad software, San Diego California, USA). Non-parametric ANOVA (Kruskall–Wallis test with Dunn's multiple comparison test) was used to compare the mean values of the intensity of the palate fullness among the different maltodextrin-beer samples. The threshold concentration values were compared using one-way analysis of variance (ANOVA) with Bonferroni's multiple comparison test. Friedman'stest (non-parametric repeated measures ANOVA with Dunn's multiple comparison test) was used to compare the values of the ranks of the intensity of the palate fullness between the different commercial beers. Non-parametric ANOVA (Kruskall–Wallis Test with Dunn's multiple comparison test) was used to compare the values of the intensities of the palate fullness of the experimental beers according to the variation of the initial temperature of the mashing process. Kruskal–Wallis test (non-parametric ANOVA) was performed to compare the molar weight distributions of the different commercial pilsner beers. Spearman's rank correlation was used to evaluate the associations between the molar weight distributions with the intensity of the palate fullness of the beers. Repeated measures analysis of variances (Repeated ANOVA) with Tukey–Kramer multiple comparisons post-test was used to compare the differences between the molar weight distributions according to the distinct mashing procedures.

Results

MWD from different commercial pilsner beer

An amount of nine Pilsner beers with similar content of alcohol and wort extract were selected for this trial (Table 2), and the determination of the MWD was carried out by AF4/MALLS/RI as described previously. The results are shown in Fig. 2. It was found that the MWD of the beers ranged between 3 and 13 kDa. Regardless of their

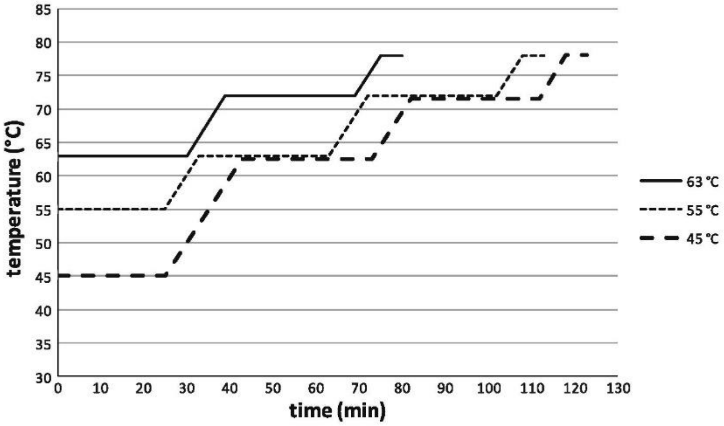

Fig. 1 Time evolution of the mashing process according to different initial temperatures

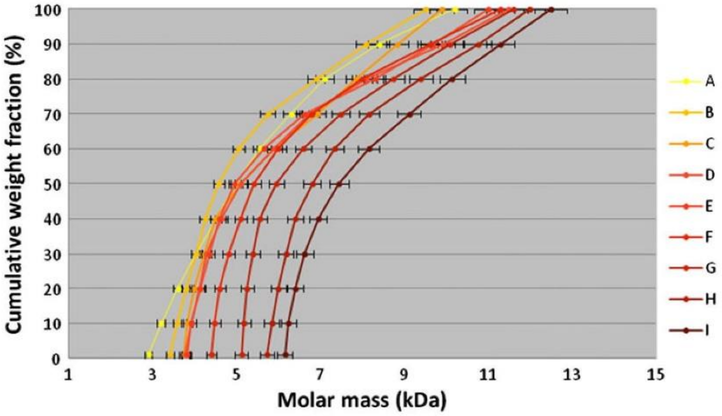

Fig. 2 Molecular weight distribution of different commercial pilsner beers

similar extract content, the range of the MWD differed significantly (Kruskal–Wallis Statistic: KW = 19.169; ρ = 0.0140) within the distinct selected beers.

MWD of standard commercial maltodextrins

In order to evaluate at which range of MWD of maltodextrins corresponded those observed previously for the commercial beers, five different types of commercial maltodextrins varying in their range of MWD were selected.

MWD of the water-maltodextrin samples

The results of this study showed that standard maltodextrins (dissolved in water) varied in a wide range of MWD (1.8–105 kDa). It can be observed in Fig. 3 that the MWD differed among the different types of maltodextrins. A good reproducibility of the values was also confirmed (standard deviation of the three samples for each type of maltodextrin). Moreover, Table 3 showed that larger elution volumes obtained by AF4 analysis corresponded to increasing ranges of MWD, indicating that the application of AF4 technique [8] was suitable for the separation of maltodextrins at this range of MWD.

MWD of the beer-maltodextrin samples

The range of MWD of different maltodextrins dissolved in samples of the same pilsner beer (20 g/L) was determined. The results are shown in Fig. 4. It can be observed that the range of MWD among the maltodextrin + beer samples varied noticeably according to the type of maltodextrin added to the sample of beer. Also, and similar to the results obtained when the maltodextrins were dissolved in water,

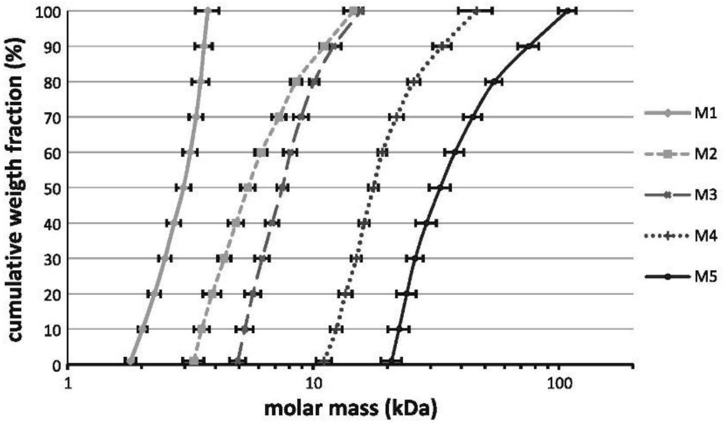

Fig. 3 Molecular weight distributions of the different commercial maltodextrins

Table 3 Molecular weight distribution and elution volumes of different commercial maltodextrins

Maltodextrine	Elutions volumen (ml)	Range of MWD (kDa)
M1	13.5–17.2	1.8–4.2
M2	15.2–23.8	2.9–14.9
M3	16.3–25.1	4.7–16.2
M4	23.6–31.0	10.5–53
M5	26.3–35.5	19–105

the elution volumes increased according to the range of MWD (Table 3). Thus, the lower MWD (2.7–8.9 kDa) corresponded to the smaller elution time (14.1–17.6 min), whereas the higher MWD (26.5–108 kDa) corresponded to the largest elution time (27.1–36.2 min) (Table 4). The results showed that the MWD of the same standard maltodextrin, dissolved in beer was slightly greater than the observed when the corresponding maltodextrin, at a similar concentration, was dissolved in water. This effect may be due to the presence of other components of the pilsner beer which may elute together with each type of maltodextrin. It is important to point out that MWD of the commercial beers (3–13KDa) were similar to those found for maltodextrins with intermediated ranges of MWD (3.4–22.3 KDa).

Palate fullness of the spiking trial

The intensity of the palate fullness according to the range of MWD and concentration of the maltodextrin-beer samples were determined (Table 5). As explained above, a preliminary test (at adequate conditions of temperature and beer sparkling) was carried out. In this trial, a comparison of the palate fullness between the different samples of beer-maltodextrin was performed when a high concentration of maltodextrin (40 g/L) was added to a pilsner beer sample.

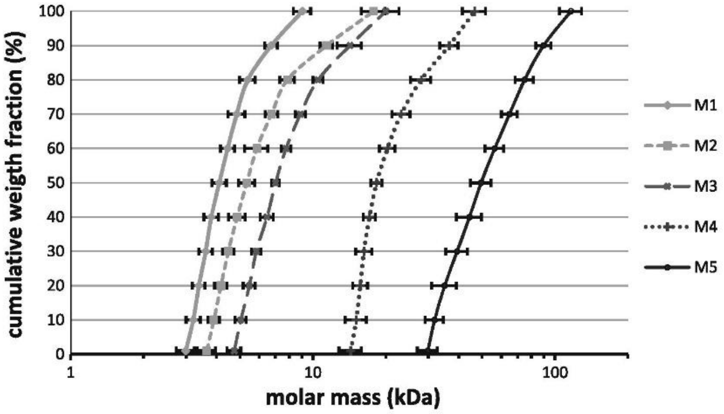

Fig. 4 Molecular weight distribution of different commercial maltodextrins in beer

Table 4 Molecular weight distribution and elution volumes of maltodextrin–beer samples

Maltodextrine	Elutions volumen (ml)	Range of MWD (kDa)
M1	14.1–17.6	2.7–8.9
M2	14.7–25.8	3.4–21
M3	15.3–27.2	4.5–22.3
M4	22.8–32.4	12.3–51
M5	25.3–36.2	26.5–108

At this concentration, strong palate fullness was perceived by the panel for all types of maltodextrins. Moreover, a slight increase ($p > 0.005$) in the intensity of the palate fullness was perceived in those samples corresponding to the higher range of MWD compared to those with the lower ranges of MWD. Regardless of the intensity of the palate fullness, the panel stated that the characteristic flavor of beer was maintained in all the samples tasted. Under these conditions, we proceeded to study the possible differences in the palate fullness according to the type of maltodextrin at different concentrations of maltodextrin-beer sample using the same approach.

Differences on the intensity of the palate fullness among distinct maltodextrin-beer samples

Table 5 shows the mean ± standard deviation of the values of the intensity of the palate fullness of different maltodextrin-beer samples, according to the maltodextrin's range of MWD and maltodextrin concentrations of the samples. It was found that at a concentration of 2.5 g/L there were slight significant differences on the intensity of the palate fullness within the samples according to the range of MWD (Kruskall Wallis statistic: 29.34, $p < 0.05$). Noteworthy, a significant difference ($p < 0.005$) on the palate fullness was reported only for maltodextrins with the higher ranges of MWD (M4 and M5) with respect to that of the lower range of MWD (M1). At a concentration of 5 g/L, also an important difference on the intensity of the palate fullness within the samples was reported (Kruskall Wallis statistic: 39.59, $p < 0.005$). At this concentration, greater differences ($p < 0.0001$) were appreciated among the samples with lower ranges of MWD (M1 and M2) compared with those with the higher ranges of MWD (M4 and M5), and also a slight significant difference ($p < 0.005$) was found among samples with low ranges of MWD (M1 and M2) compared with that of intermediated range of MWD (M3). At a concentration of 10 g/L, the differences in the palate fullness within the beer samples were more noticeable (Kruskall Wallis statistic: 56.89, $p < 0.0001$). In addition, a great increase in the intensity of the palate fullness was perceived in those samples corresponding to the higher MWD (M4 and M5) with respect to those samples with the lower MWD (M1 and M2). Also, it was perceived an increase ($p < 0.005$) in the sample with intermediate palate fullness (M3) with respect to those with the lower palate fullness (M1 and M2). The same pattern was reported at a concentration of 20 g/L (Kruskall–Wallis statistic: 69.63, $p < 0.0001$). As mentioned above, at 40 g/L, the palate fullness was perceived as strong in all the samples tasted. Even though a significant difference within the samples was still reported (Kruskall–Wallis statistic: 34.61, $p < 0.005$), only those samples corresponding to the higher range of MWD (M4 and M5) showed a significant ($p < 0.005$) increase in the palate fullness. At a

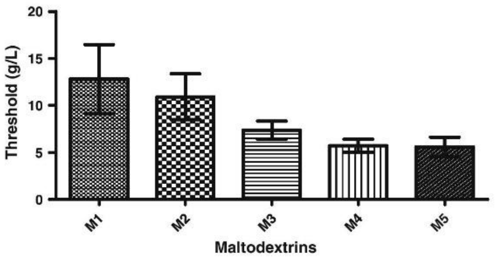

Fig. 5 Threshold concentration values of maltodextrins in the spiking trial. Mean ± standard deviation of the threshold concentration values for each maltodextrin, defined as the concentration value by which the palate fullness was perceived as different with respect to the control pilsner beer (without maltodextrin)

Table 5 Intensity of the palate fullness of the beer samples according to the concentration and the range of molecular weight distribution of maltodextrins

Maltodextrin (kda)	Mean ± standard deviation of the intensity of Palate Fullness (n = 32)						
	Control	2.5 g/L	5.0 g/L	10.0 g/L	20.0 g/L	40.0 g/L	80.0 g/L
M1 (2.7–8.9)	2.78 ± 0.418	3.00 ± 0.33	3.31 ± 0.47	3.94 ± 0.404	4.26 ± 0,452	4.68 ± 0.47	6.52 ± 0.51
M2 (3.4–2.1)	2.76 ± 0.43	3.23 ± 0.43	3.52 ± 0.51	4.14 ± 0.35	4.38 ± 0.49	5.38 ± 0.50	6.57 ± 0.50
M3 (4.5–22.3)	2.86 ± 0.51	3.53 ± 0.51	4.33 ± 0.61	4.93 ± 0.59	5.60 ± 0.63	6.13 ± 0.51	6.66 ± 0.48
M4 (12.3–51)	2.89 ± 0.56	3.63 ± 0.49	4.52 ± 0.51	5.15 ± 0.50	5.63 ± 0.49	6.73 ± 0.45	7.00 ± 0.00
M5 (26.5–108)	3.21 ± 0.53	4.05 ± 0.62	4.73 ± 0.56	5.21 ± 0.53	6.63 ± 0.49	7.00 ± 0.00	7.00 ± 0.00

concentration of 80 g/L, an extremely strong palate fullness was perceived for all the samples such that differences with respect to the range of MWD were not appreciated by the panel.

Threshold concentration of maltodextrins

The mean ± standard deviation of the threshold concentration of the maltodextrin-beer samples are shown in Fig. 5. It was found that for the samples with the lower ranges of MWD: 2.7–8.9 kDa, the threshold concentration by which the palate fullness was perceived as different with respect to the control standard pilsner was significantly higher than the threshold concentration corresponding to the higher ranges of MWD: 12.3–51 kDa (Bonferroni's multiple test: $t = 3.609$, $p < 0.0001$) and 26.5–108 kDa (Bonferroni's multiple test: $t = 3.601$, $p < 0.0001$). A slightly significant difference was also observed between the threshold concentration of the sample with the lowest range of MWD: 2.7–8.9 with that of intermediated MWD: 4.5–22.3 (Bonferroni's multiple test: $t = 3.06$, $p < 0.05$).

Palate fullness of the selected commercial pilsner beers

Table 6 shows the individual ranks of each beer arranged and labelled in an ascending order according to the intensity of the palate fullness by the tasting panel and the total rank obtained from the sum of the individual ranks for each beer. It was found that the ranks of the intensity of the palate fullness within pilsner beers differed significantly (Friedman statistic, Fr: 68.397, $p < 0.0001$). Applying Dunn's multiple test for individual comparisons, it was found that beers A and B exhibited significant lower ranks of palate fullness compared to beers C, D and E ($p < 0.005$), being this difference extremely different between beers A and B compared to beers F, G, H and I ($p < 0.0001$). Also there was a slight significant difference between beer E with beers H and I ($p < 0.005$). There were no differences between beers A and B as well as among beers F, G, H and I. Figure 6 shows the range of MWD of the different beers arranged according to the intensity of the palate fullness by the panel (1-9). The range of MWD was determined from different points (1, 50, 70, 85, 100 %) of the cumulative weight fraction calculated for each beer sample (Fig. 2). Significant correlations were found between the increase in the range of the molecular weight distribution and the rank of the intensity of the palate fullness ($r = 0.8667$, Spearman $\rho = 0.0045$ at a cumulative weight fraction of 50 %; $r = 0.8443$, Spearman $\rho = 0.0052$ at a cumulative weight fraction of 70 %, $r = 0.8234$, Spearman $\rho = 0.0007$ at a cumulative weight fraction of 85 % and $r = 0.8356$, Spearman $\rho = 0.002$ at a cumulative weight fraction of 100 %). Also it can be seen in the figure that those molecular weight distributions corresponding to 50, 70 and 100 % from the cumulative weight fraction were more associated with the increase in the palate fullness of the beers.

Effect of technological aspects of brewing on the MWD and the palate fullness of experimental beers

The possible influence of differences in the mashing process and/or the quality of the raw material on the variation of the range of MWD of beer was studied. Also it was evaluated whether these variations were perceived as differences on the palate fullness by the tasting panel. Table 7 shows the associations of the ranges of MWD with the palate fullness of the beers. The resulting beers were obtained using malted barley with a Kolbach Index of 41 % and mashing procedures initiated at different temperatures (45, 55, 63 °C). It can be observed that significant higher ranges of MWD were obtained when the mashing process was initiated at 63 °C compare to that obtained when the mashing process was initiated at 45 °C (Tukey–

Table 6 Intensity rank of the palate fullness of different commercial pilsner beer according to ten different tastings

BEERS	Intensity rank of palate fullness										Sum	Rank
	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10		
A	1	2	3	1	3	1	2	2	1	3	19	2
B	2	1	1	2	1	2	1	1	3	1	15	1
C	7	4	7	6	5	4	5	6	4	6	54	5
D	4	5	2	3	2	3	4	3	2	3	31	3
E	3	3	6	4	4	5	3	5	6	4	43	4
F	6	6	4	7	8	7	7	4	7	5	61	6
G	8	7	8	5	7	8	9	7	8	8	75	8
H	5	8	5	8	6	6	6	8	5	7	64	7
I	9	9	9	9	9	9	8	9	9	9	89	9

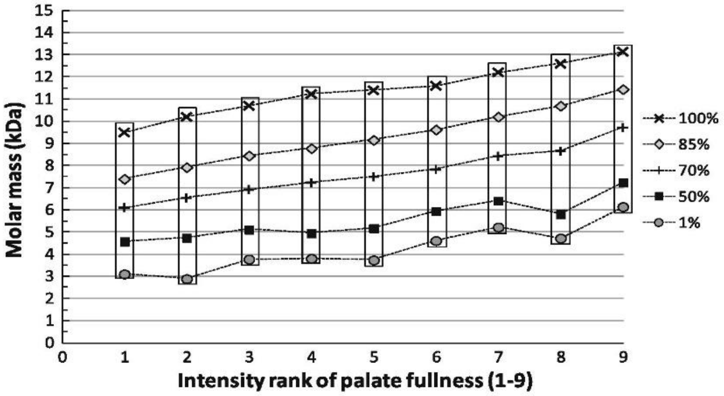

Fig. 6 Association of the molecular weight distribution with the intensity rank of the palate fullness of different commercial pilsner beers. Range of MWD of the different beers arranged according to the intensity of the palate fullness. The range of MWD was calculated from 5 different points of the cumulative weight fraction determined for each beer sample

Kramer test: $q = 9.516$, $p < 0.001$) or 55 °C (Tukey–Kramer test: $q = 7.018$, $p < 0.001$). Also, there were significant differences on the intensity of the palate fullness according to the variation of the initial temperature of the mashing process (Kruskal–Wallis statistic KW = 252.41). Therefore, beers produced at an initial temperature of 63 °C exhibited significantly stronger palate fullness compared to those produced at initial temperatures of 45 °C ($p < 0.0001$) and at 55 °C ($p < 0.0001$) (Table 7). There were no statistical differences on the MWD and on the intensity of the palate fullness using initial temperatures of 45 °C compared to those obtained using 55 °C. Table 8 shows the associations of the ranges of MWD with the palate fullness of the resulting beers according to the trials carried out using barley malted with a Kolbach Index of 36 % and at different initial temperatures (45, 55, 63 °C). Higher ranges of MWD were obtained when the mashing process was initiated at 55 and 63 °C compared to those obtained using a temperature of 45 °C (Tukey–Kramer test: $q = 13.523$, $p < 0.0001$ between 45 and 55 °C; $q = 12.730$, $p < 0.0001$ between 45 and 63 °C). There were no statistical differences among the range of MWD using initial temperatures of 55 °C compared with those observed using initial temperatures of 63 °C. It was found that those beers elaborated at initial temperatures of 55 and 63 °C showed a tendency to be perceived with greater palate fullness than those elaborated with initial temperatures of 45 °C, although a significant difference on the palate fullness was not appreciated.

Table 7 Range of molecular weight distribution and intensity of the palate fullness of experimental beers (Kolbach Index: 41 %) according to different initial temperatures of the mashing process

Beer	Initial Temp of Masching (°C)	Range of MWD (kDa)	Intensity of palate fullness (1–7) (mean ± SD) n = 32
1	45	1.84–6.89	2.70 ± 0.59
2	45	1.76–7.13	2.66 ± 0.60
3	45	1.92–7.09	2.28 ± 0.46
4	45	1.68–7.23	2.39 ± 0.49
5	55	2.03–7.47	3.10 ± 0.66
6	55	2.11–7.72	3.30 ± 0.65
7	55	2.08–8.13	3.32 ± 0.47
8	55	2.23–7.93	3.67 ± 0.54
9	63	2.74–10.02	4.56 ± 0.50
10	63	3.04–10.84	4.60 ± 0.72
11	63	2.89–11.57	4.53 ± 0.57
12	63	3.31–11.13	5.14 ± 0.65

Discussion

The structural properties of beer components play an important role in the determination of the palate fullness. However, the quantitative associations of structural properties of dextrins as well as from other beer components with the palate fullness have not yet been well elucidated. Early studies carried out by Vermeylen [28] have shown a relationship between wort dextrin concentration and beer palate fullness. On the other hand, studies carried out by Ragot et al. [29] have shown that a concentration of 50 g/L of maltodextrin must be added to a light beer to produce a detectable increase in viscosity and thus to enhance significantly its palate fullness. Because the natural content of dextrins in a beer ranges from 10 to 50 g/L, the authors concluded that dextrins alone do not account for the viscosity or thickness of beer. In contrast, in this work, the results of the spiking trial indicated that the effect of the maltodextrin concentration on the intensity of the palate fullness was influenced by the range of MWD of the maltodextrins. Nevertheless, this effect could be appreciated only when intermediated concentrations of

Table 8 Range of molecular weight distribution and intensity of the palate fullness of experimental beers (Kolbach index: 36 %) according to different initial temperatures of the mashing process

Beer	Initial temp of mashing (°C)	Molecular weight distribution (kDa)	Intensity of palate fullness (1-7) (mean ± SD) n = 32
1	45	3.12–9.46	4.20 ± 0.47
2	45	3.21–9.18	4.44 ± 0.50
3	45	3.51–10.13	4.10 ± 0.62
4	45	2.97–9.31	4.42 ± 0.57
5	55	4.05–11.75	4.50 ± 0.50
6	55	4.59–12.5	4.64 ± 0.54
7	55	3.98–12.1	4.67 ± 0.61
8	55	4.25–12.6	4.75 ± 0.58
9	63	4.13–12.07	5.17 ± 0.52
10	63	4.29–12.6	5.26 ± 0.51
11	63	4.91–12.26	5.17 ± 0.61
12	63	4.72–12.72	5.25 ± 0.58

maltodextrins (5–20 g/L) were added to the beer samples. When lower concentrations (2.5 g/L) of maltodextrins were added, only small differences could be perceived on the intensity of the palate fullness (according to the range of MWD), whereas by the addition of higher concentrations (40–80 g/L), the intensity of the palate fullness was too strong so that the influence of the range of MWD could not be clearly appreciated. Moreover, the threshold concentration by which the palate fullness was perceived as different with respect to a control pilsner beer was also found to be highly dependent on the range of MWD of the maltodextrins. These results suggest that the range of MWD plays an important role in the production of a beer with a defined palate fullness.

The range of MWD varied significantly among the commercial beers despite their similar alcohol and wort extract content, suggesting a role of technological parameters of the brewing process in the variation of the range of the MWD of beer components as discussed below. It is important to point out that the values of the intensity of palate fullness obtained by the addition of maltodextrins of intermediated ranges of MWD in the spiking trial corresponded to those observed for the commercial beer samples, suggesting that pilsner beers contain dextrins of intermediated MWD (3.4–22.3 kDa). It is well known that dextrins are the major components (75–80 %) of the residual unfermented solids present in the beer [4, 5, 18]; however, nitrogenous compounds in a minor proportion (6–9 %) and β-glucans in very low quantities [4, 15, 29] may also contribute to the MWD of the final beverage. The variations observed in the range of MWD of the beers strongly reflected the differences in the intensity of the palate fullness perceived by the panel. In addition, it was found that variation of technological parameters of the brewing process also influenced the range of MWD of experimental beers. Thereby, using a barley malt with a Kolbach index of 41 %, significant higher ranges of MWD were obtained when the mashing process was initiated at 63 °C compared to those obtained when the mashing process was initiated at 45 or 55 °C. As expected, these beers also exhibited stronger palate fullness. It is possible that at these initial temperatures (45 or 55 °C) occurs an intense mashing process, favoring a better degradation of proteins and β-glucans. Also partial degradation of starch by amylases may occur [8]. On the other hand, gelatinization of the starch granules of this malted barley was found to occur at 62.4 °C. At this temperature, the crystalline structure of starch is broken rendering glycosidic bonds accessible to the amylases [8]. However, at 65 °C the activity of the β-amylase decreases rapidly whereas the α-amylase activity is favored, leading to the production of dextrins with high molar masses [30, 31] which may contribute to a higher range of molecular weight distribution of the total carbohydrates of the final beer. Also it must be considered that during malting temperatures can reach up to 80 °C [6, 32, 33]. This also causes partial breaking of the crystalline structure of the starch granule [8, 30, 31], allowing further a slow degradation of starch at temperatures lower than gelatinization temperature, during the mashing process. In contrast, using barley malted with a Kolbach index of 36 % and at different initial temperatures (45, 55, 63 °C) of mashing process, it was shown that higher ranges of MWD were obtained when the mashing process was initiated at 55 and 63 °C compared to those obtained using a temperature of 45°, while there were no differences using initial temperatures of 55 °C compared with those observed using initial temperatures of 63 °C. Because these experiments were performed using malted barley with a low degree of modification, the cellular wall of the starch granule would not be degraded completely during the process of malting [8, 30, 31], therefore hindering the access of the enzymes to the granule, at this range of temperature. In contrast, at 45 °C, the activity of proteases and glucanases intensively degrades the cellular wall of the granules during the mashing process [30, 31], allowing a better action of amylases, thus leading to the presence of carbohydrates of lower ranges of MWD in the final beer. According to the sensory analysis, beers produced with malted barley with a Kolbach index of 36 % exhibited a stronger palate fullness compared with those elaborated with a Kolbach index of 41 %. Among those produced with malted barley with a Kolbach index of 36 %, those elaborated at initial temperatures of 55 and 63 °C showed a tendency to be perceived with greater palate fullness than those elaborated with initial temperatures of 45 °C, although a significant difference on the palate fullness was not appreciated. In contrast, a great difference on the intensity of the palate fullness of the final beer was perceived according to the initial temperature of the mashing process using barley with a Kolbach index of 41 %. These results demonstrate that variations in the initial temperature during the mashing process influenced the range of MWD of the beer, through the modulation of the access of the amylases to the glycosidic bonds, thus leading to the production of dextrins of different MWD which in turn would determine variations in the MWD of the total carbohydrate content. Although other components such as proteins of low molecular weight contribute to the MWD [27], it must be considered that the carbohydrate content (30 g/L) of the beer is around sixfold the content of proteins (5 g/L). Therefore, our results suggested that dextrins would be an important contributor to the range of MWD of the beer, thus influencing the intensity of the palate fullness of the beer.

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Determination of the influence of starch sources and mashing procedures on the range of the molecular weight distribution of beer using field-flow fractionation

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To produce experimental beers, different mash mixtures (barley malt, barley malt + 30% pre-cooked maize, barley malt + 30% nonmalted spelt) and distinct mashing procedures (infusion and decoction) with variations of the rest time and initial temperatures were evaluated. The range of molecular weight distribution (MWD) of the resulting beers was determined using asymmetrical flow field flow fractionation coupled to multiangle laser light scattering and refractive index. There were no differences on the range of MWD among the beers, according to infusion or decoction, using similar raw materials and initial temperatures (45 and 55°C). However the range of MWD was higher ($p < 0.005$) when using infusion at an initial temperature of 63°C, regardless of the raw material. The use of maize did not alter structural properties of the beer, while mash containing nonmalting spelt caused an elevation on the MWD ($p < 0.001$) and a lower ($p < 0.05$) apparent degree of fermentation. Therefore the range of the MWD of the beers was influenced by the quality of the raw material and the initial mashing temperature, whereas apparent degree of fermentation values were affected only by the type of starch source. Thus the determination of the MWD is an important tool for monitoring the production of beer. Copyright © 2013 The Institute of Brewing & Distilling

Keywords: molecular weight distribution; asymmetrical flow field flow fractionation; multiangle laser light scattering; refractive index; mash; temperature

Introduction

The main goal of the brewing process is the degradation of soluble starch to fermentable sugars and then the production of alcohol through yeast metabolism. Water, malt, hops and yeast are the four main ingredients for manufacturing beer. Malted barley, which generates high levels of amylolytic enzyme activity during germination, is the most used source of carbohydrates in the brewing process. Nevertheless wheat, rye, triticale spelt and maize corn are also suitable for brewing and they are commonly added to barley malt as adjuncts *(1–3)*. Starch granules from different adjuncts vary in shape, size and amylose/amylopectin ratio. Physical and chemical properties of starch such as viscosity, shear resistance, gelatinization and retrogradation, among others, are functions of their amylose/amylopectin ratio *(4)*. Also, according to their amylose and amylopectin content, starch samples contain a vast range of molecular weight and size distributions, which may determine rheological and more general technological properties of the polymer that are important for commercial processing *(4)*. Trials using alternative cereals and pseudo-cereals such as sorghum have been assessed in brewing, resulting in beers with novel sensory properties *(5–7)*.

Degradation of carbohydrates, β-glucans and proteins is determined by the activity of different enzymes (amylases, β-glucanases and proteases) during mashing. The process of mashing consists of mixing and heating the mash in the mash tun, and can be carried out alternatively by infusion, decoction or a combination of the two processes. Infusion mashing indicates only enzymatic digestion at different temperatures and rests. On the other hand, decoction mashing includes additional thermal degradation. The main parameters of this process are temperature, pH, time and wort concentration. Thermal stability of starch-hydrolysing enzymes, particularly for β-amylase, is critical for the fermentable sugar yield during mashing and so determines the final carbohydrate composition of the resulting beer. In fact, the mashing temperature profile is a balance between the temperature required for starch gelatinization needed to enable efficient hydrolysis and the rate of thermal inactivation of the amylases *(8)*. The lowest temperature (40–45°C) is the optimal temperature for cell wall degrading enzymes (β-glucanases). The proteases work best between 45 and 50°C, the β-amylase between 60 and 65°C and the α-amylase between 65 and 75°C *(8)*. α-Amylase breaks large molecules of amylose and amylopectin of the starch granule into dextrins molecules of lower molecular weight *(4,9)*, thereby allowing rapid degradation of the granules during the mashing process. Further, β-amylase sequentially removes units of maltose from the nonreducing end of large dextrins. Proteases reduce proteins to small peptides and amino acids. Therefore, the final composition of beer includes small residual fermentable sugars, a variable amount of dextrins and small quantities of low molecular weight peptides that contribute to the molecular

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weight distribution (MWD) of the beer *(10)*. Previous reports confirmed that the MWD of the beer components is an important structural characteristic influencing sensory properties such as palate fullness *(10)*. In this work we used a new analytical approach (AF4/MALLS/RI) to evaluate the influence of the type of mashing (infusion or decoction), as well as variations on the mashing parameters (temperature and rest time), and the use of different adjuncts (pre-cooked maize and nonmalted spelt) on the range of the MWD of the resulting experimental beers.

Materials and methods

Production of the experimental beers

Experimental beers were produced on a 10 L scale according to standard protocols *(11)*. The procedures were carried out using barley malt with a Kolbach index (KI) of 41 (variety Marthe, Saaten Union, Germany) as standard raw material. Additionally two different adjunct mixtures were used: *(1)* barley malt mixed with commercial dry pre-cooked maize cornmeal (Harina Pan, Alimentos Polar CA, Colombia with 78% of total carbohydrate, 8% of protein and 1.5% of fat) at 30%; and *(2)* barley malt mixed with nonmalted spelt (dehulled spelt, variety Zollernspelz, Saaten Union, Isernhagen, Germany) at 30%. For the standard barley malt, as well as for the two adjunct mixtures, the mashing was carried out using infusion or decoction (one and two steps) method described elsewhere. For the infusion method, initial temperatures of the mashing process were tested at 45, 55 and 63°C (Fig. 1). In addition, when using an initial temperature of 63°C, reductions of the rest time from the standard 30 min to 15 and 1 min were also tested, as indicated in the figure. Following the above mentioned rest times at 63°C, the mash was heated (1°C/min) to reach a temperature of 72°C, and then following a rest time of 30 min, the mash was heated again (1°C/min) to reach a temperature of 78°C. At this temperature (78°C) and after a rest time of 5 min, the process of lautering was carried out. Similarly, for those processes started at 55 and 45°C, after a rest time of 25 min, the samples were heated (1°C/min) to reach a temperature of 63°C (rest time of 30 min), then the mash was heated (1°C/min) to a temperature of 72°C (rest time of 30 min) and 78°C successively. For the decoction method, one- and two-step decoction processes were carried out at initial temperatures of 45 and 55°C, respectively (Fig. 2). Around one-third of the main mash (6 L) was removed to a separate vessel, which was heated to 95°C for 10 min, and returned to the main mash (Fig. 2). Further phases of the brewing process (wort boiling, wort filtration, fermentation, beer filtration and beer filling storage) were performed in an identical manner according to standard procedures *(10–13)*. Briefly, wort boiling was carried out for 80 min (98–99°C). Primary fermentation was performed at a temperature of 12°C for 6 days and secondary fermentation at a temperature of 15°C and at a pressure of 1 bar for 3 days. Maturation was performed for 3 weeks at a temperature of 0°C. The resulting beers were stored at 2°C. For good reproducibility of the results, experiments were carried out four times for each mashing procedure and mash composition (standard and adjuncts mixtures). For 10 L of wort, 1.05 kg of barley malt and 0.45 kg of the correspondent cereal adjunct (maize or spelt) were required. Thus, the final original gravity for each beer was of around 11.7% w/v. For all trials, the same quantity of hops was used during wort boiling, resulting in around 20 IBU of bitterness for each type of final beer.

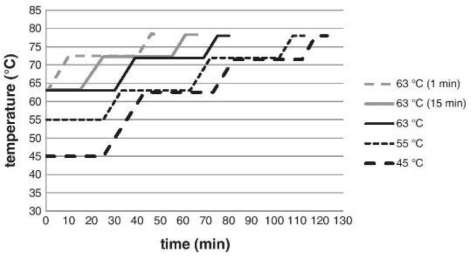


Figure 1. Time evolution of infusion procedures.

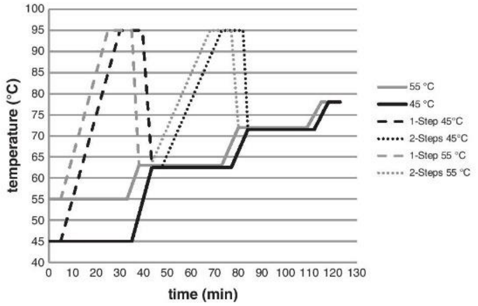


Figure 2. Time evolution of decoction procedures.

Structural properties

Structural properties of the different starch sources (standard barley malt KI 41, and adjunct mixtures) in the mash as well as of the experimental beers were determined by asymmetrical flow field flow fractionation (AF4; Wyatt Technology, Germany) coupled to multiangle laser light scattering (MALLS; Dawn, Heleos II, Wyatt Technology, Germany) and refractive index (RI) as the quantitative detector (Agilent Series 1200 G1362A, Agilent Technologies, Germany) *(4,14)*.

Structural properties of the mash samples from different starch sources. AF4/MALLS/RI, described in a previous work *(15,16)*, was used to determine the weight average molar mass (Mw), MWD and root mean square (rms) radius of mash samples (standard barley malt, barley malt + 30% pre-cooked maize, barley malt + 30% nonmalted spelt) under the same experimental conditions. The inserted spacer had a height of 350 μm, a width of 21.5 mm at the widest position, approximately where the sample was focused (270 s), and a long channel of 240 mm. The samples were dissolved (10 g L^{-1}) in distilled water at 65° C, by stirring for 1 min, then the samples were heated to reach a temperature of 85°C (5 min) and they were stirred for 5 min at 85°C. Further, they were cooled to 20°C and filtered. Aliquots (50 μL) of the resulting samples were injected in the channel. Measurement was carried out at 25°C. Carrier solvent was composed of 50 mM sodium nitrate and 250 ppm sodium azide in Millipore water. Separation was conducted with a 5 kDa membrane (Nadir Regenerated Cellulose, Wyatt Technology, Germany). Injection flow was of 0.2 mL/min. The elution flow was 1 mL/min and the cross flow was decreased from 3 to 0.1 mL/min within 30 min. Then, cross flow was constant for 20 min at 0.1 mL/min and finally reduced to zero. The intensity of scattered light was measured simultaneously at 18 different scattering angles ranging from 10 to 160°. A d*n*/d*c* (variation of the refractive index in a solution) of 0.146 mL g^{-1} was used to determine concentration of polysaccharides in aqueous solvents by RI detector. Baselines of RI detection were subtracted by baselines from blank runs, since RI baselines are influenced by different salt and pressure conditions during a cross flow gradient in AF4. Pullulan standards (1 g L^{-1}; PSS GmbH, Germany) were used for the calibration of the detectors (Table 1). Obtained data were processed by Astra version 6.0.3.16 software (Wyatt Technology, Germany).

Determination of the range of the MWD of the experimental beers. The determination of the MWD of the experimental beers was carried out using AF4/MALLS/RI as described previously *(10)*. Briefly, carbon dioxide was removed from beers through a filtration process before AF4 analysis and 100 μL of each resulting experimental beer was injected. For this purpose, the inserted spacer was of 490 μm height and the width of 21.5 mm at the widest position. Time focusing in the long channel was 500 s. Separation was conducted with a 1 kDa membrane (Polyethersulfone, Wyatt Technology, Germany). Elution flow was 1 mL min^{-1} and cross flow was decreased from 4 to 0.1 mL min^{-1} for 30 min. Then cross flow was constant for 20 min at 0.1 mL min^{-1} and finally reduced to zero. The d*n*/d*c* was of 0.146 mL g^{-1}. The MWD, expressed as the cumulative weight fraction as a function of the molar mass, was obtained using the Astra version 6.0.3.16 software (Wyatt Technology, Germany).

Protein determination

Total nitrogen was determined using the Kjeldahl method according to MEBAK procedures *(17)*.

Determination of the degree of fermentation

Original gravity and apparent degree of fermentation were determined using an Alcolyzer Plus (Alcolyzer beer analysing System, Anton Paar).

Statistics

Statistical analysis was carried out using Graph Pad InStat version 3.05 (Graph Pad software, San Diego, CA, USA). Repeated measures analysis of variance (repeated ANOVA) with Tukey–Kramer multiple comparisons post-test *(18)* was used to compare the differences between the values of original gravity, apparent degree of fermentation and nitrogenous compounds owing to variations of the mashing procedure. According to this test, the differences were considered as significant if the value of the *q*-ratio was $>$4.523 and the *p*-value was $<$0.05 *(18)*. The values of *p* and the 95% confidence interval for each comparison are shown in the results. One-way ANOVA was applied to compare the range of MWD obtained for each mashing procedure. The minimum and maximum values, as well as the values of the median, mean, standard deviation and the 95% confidence interval of each range of MWD were reported. Variations of the range of MWD, according to mashing procedures, were compared using the Friedman Test with Dunn post test *(19)* with a significance level (α) of 0.05.

Results

Characteristics of the mixtures of starch sources in the mash

The structural properties of the mash samples were determined using AF4/MALLS/RI/. Figure 3 shows that, for the same range of molar weights (1.0×10^6 to 1.0×10^7 g mol^{-1}), the mixture of malted barley + nonmalted spelt exhibited significant greater rms values (150–210 nm) compared with those observed for barley malt (90–145 nm), and particularly for the mixture of barley malt + pre-cooked maize (60–103 nm). This indicates that this mixture has a noticeably less compact conformation compared with that observed for the standard and for the mixture of barley malt + pre-cooked maize.

Characteristics of the experimental beers using different mashing procedures and starch sources

Original gravity. Figure 4(a) shows that the original gravity was similar for all beers (~11.7% w/v), regardless of the type of adjunct and mashing procedure, when using a standard rest time (30 min). However, a significant decrease in the original gravity values was observed at an initial temperature of 63°C and rest time of 15 min ($p < 0.05$; 95% CI 0.043–1.016 when using malted barley and $p < 0.05$, 95% CI 0.0129–1.007 when using nonmalted spelt as adjunct) and of 1 min ($p < 0.01$; 95%

Table 1. Molecular weight of standard solutions

		manufacturer	Obtained
Standard solution	Retention time (min)	Molar mass (kDa)	Molar mass (kDa)
Pullulan 6 kDa	14.1	5.9	6.8
Pullulan 10 kDa	15.7	9.6	10.2
Pullulan 21 kDa	21.0	21.1	23.5
Pullulan 47 kDa	26.9	47.1	49.4
Pullulan 107 kDa	34.6	107.0	117.6
Pullulan 200 kDa	38.4	200.0	191.3
Pullulan 708 kDa	42.8	708.0	694.9
BSA	16.5	66.5	64.8

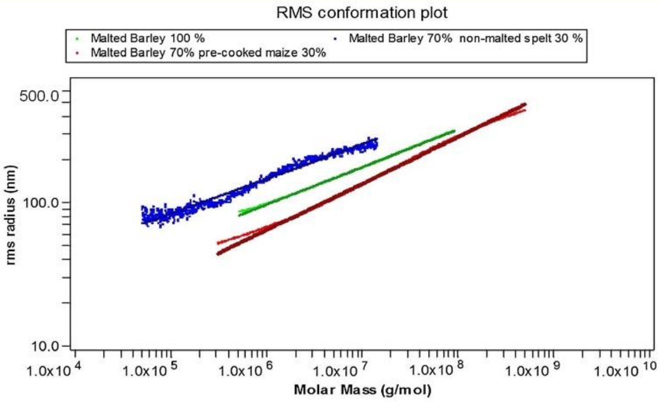


Figure 3. Structure characteristics of the different mixtures of starch sources in the mash analysed by AF4/MALLS/RI.

CI 0.223–1.196 when using malted barley and $p < 0.01$, 95% CI 0.303–1.297 when using barley malt + nonmalted spelt) compared with those obtained at a rest time of 30 min. Slightly lower values of the original gravity were observed in those beers produced using pre-cooked maize at an initial temperature of 63°C with a rest time of 1 min (Fig. 4).

Apparent degree of fermentation. Figure 4(b) shows that there were no differences in the apparent degree of fermentation (ADF) values according to the initial temperature (45 or 55°C), the mashing method (decoction or infusion) or the type of mash mixture used. Nevertheless, when using an initial temperature of 63°C, the reduction of the rest to 15 min caused a slight decrease in the ADF values. Moreover, the reduction of the rest time to 1 min caused a significant decrease in the ADF values ($p < 0.001$; 95% CI 3.896–5.664 when using malted barley; $p < 0.001$; 95% CI 6.452–8.388 when using barley malt + nonmalted spelt and $p < 0.001$; 95% CI 2.337–3.643 when using barley malt + pre-cooked maize) compared with those observed at a rest time of 30 min. It is important to point out that, when using nonmalted spelt as adjunct, all ADF values regardless of the temperature and mashing procedures were found to be under brewing standard specifications (80%).

Nitrogenous compounds. Figure 4(c) shows that, when using standard barley malt, the nitrogenous compound concentration (mg L^{-1}) was slightly elevated among those beers produced at an initial temperature of 45 and 55°C, compared with those produced at an initial temperature of 63°C. There were no differences according to the mashing method (infusion or decoction) at the same initial temperature (45 or 55°C). The decrease in the nitrogenous compound concentration observed for those beers produced at the initial temperature of 63°C was more noticeable when reducing the rest to 15 min and statistically significant when reducing the rest time to 1 min ($p < 0.001$; 95% CI 36.177–128.36). Similarly a significant decrease ($p < 0.005$; 95% CI 11.161–131.74) in this parameter was observed when using nonmalted spelt as an adjunct at a temperature of 63°C and a reduction of rest time to 1 min. Regardless of the mashing process, the addition of 30% of pre-cooked maize to malted barley decreased significantly ($p < 0.001$; 95% CI 120.6–218.3) the amount of nitrogenous compounds in the resulting beers compared with the amounts observed when beers were produced using standard malted barley (Fig. 4c).

Range of the molecular weight distributions. Figure 5 illustrates the MWD (expressed as the cumulative weight fraction as a function of the molar mass) of beers obtained according to the distinct mashing procedures produced using: (a) barley malt; (b) barley malt + nonmalted spelt; and (c) barley malt + pre-cooked maize. Each curve represents the range of the molecular weight distribution for each experimental condition. Each point of the curve (cumulative weight fraction vs molar mass) represents the mean ± standard deviation of four replicates of each beer sample.

Table 2 shows the minimum and maximum values, the values of the median, mean, standard deviation and 95% confidence interval for each range of MWD obtained using different mashing procedures and distinct mash mixtures. There were no significant differences between the ranges of the MWD among those beers produced using infusion at an initial temperature of 45°C, compared with those using one- or two-step decoction procedures. Similarly there were no differences among the ranges of MWD of those beers produced using infusion at the initial temperature of 55°C, compared with those using one- or two-step decoction. Nevertheless, there was a statistical difference ($p < 0.001$) between the range of the MWD for those beers obtained using an initial temperature of 63°C and a rest of 30 min compared with those obtained using an initial temperature of 45 or 55°C. In addition, the procedures carried out at an initial temperature of 63°C resulted in a significant elevation of the range of the MWD of the beers when using a rest time of 15 min, as well as a rest time of 1 min ($p < 0.05$ and $p < 0.001$, respectively). For those beers produced with malted barley mixed with non-malted spelt, it was observed that the elevation of the initial infusion temperature from 45 to 55°C caused a significant increase ($p < 0.05$) in the range of the MWD of the resulting beers. Moreover the use of an initial temperature of 63°C, at a rest time of 30 min, caused a great ($p < 0.001$) increase in the MWD of the beers. Also, the reduction of the rest time (at an initial temperature of 63°C) to 15 and 1 min caused a significant ($p < 0.001$) increase in the range of MWD among the beers obtained. There were no significant differences

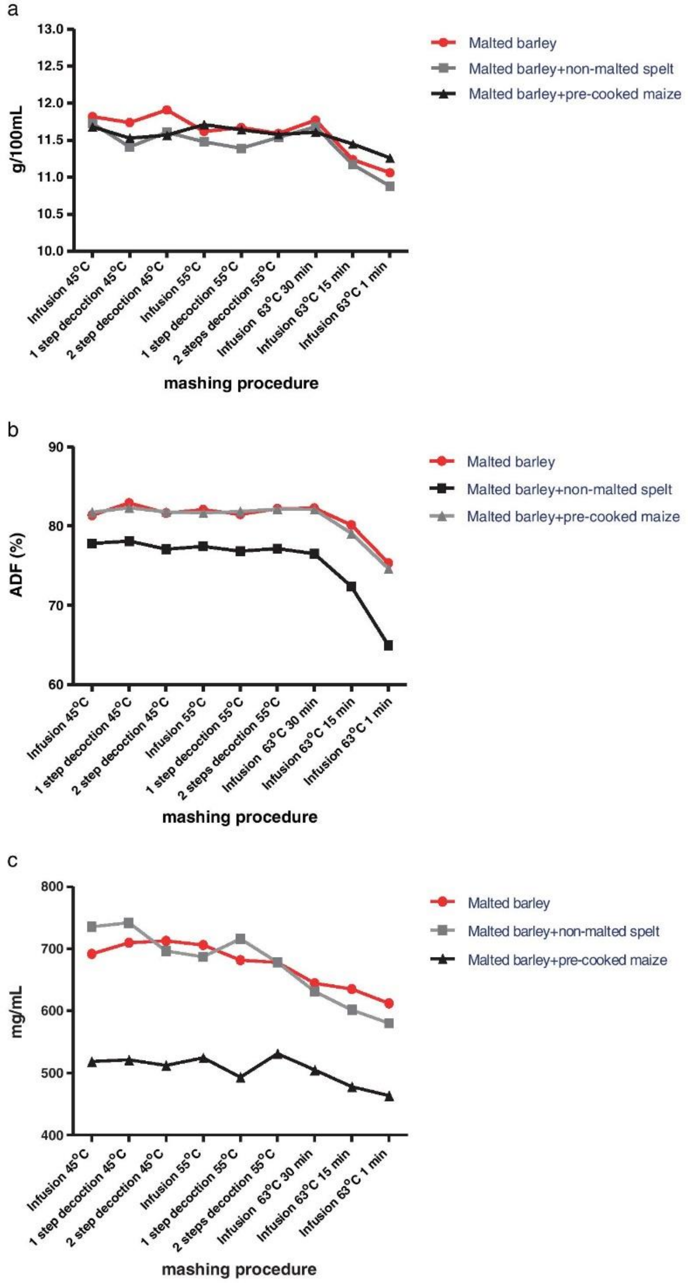
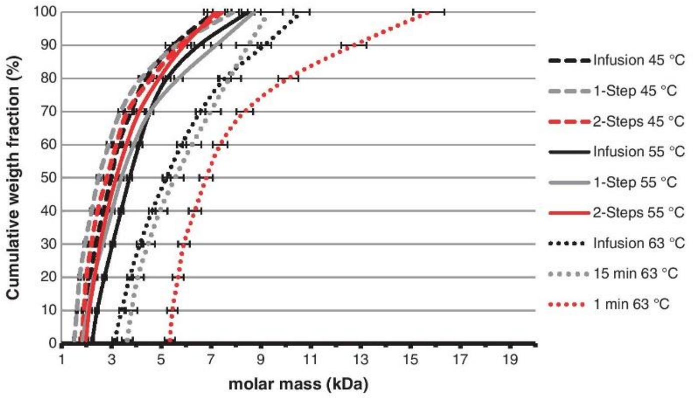


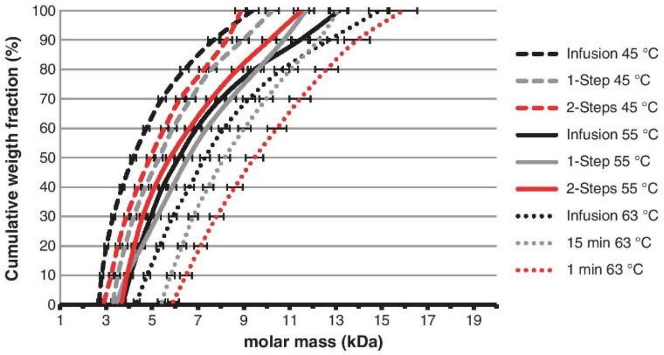
Figure 4. (a) Original gravity according to different mashing procedures; (b) apparent degree of fermentation according to different mashing procedures; (c) nitrogenous compounds in experimental beers according to different mashing procedures.

among the range of the MWD of the beers produced using one- or two-step decoction at an initial temperature of 45°C, or using one- or two-step decoction at an initial temperature of 55°C. For those beers produced with malted barley mixed with pre-cooked maize, it was found that the MWD of the beers obtained by infusion at an initial temperature of 63°C and 55°C were more elevated than that

a) Using Malted Barley



b) Using a mixture of Malted Barley and 30% non-malted spelt



C) Using a mixture of Malted Barley and 30% pre-cooked maize

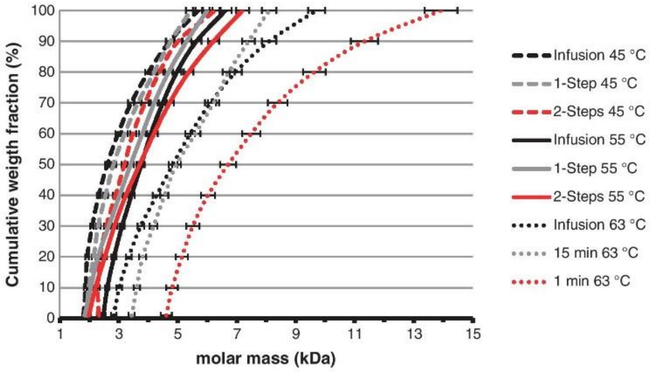


Figure 5. Molecular weight distribution of different experimental beers expressed as the cumulative weight fraction as function of the molar mass: (a) using malted barley; (b) using a mixture of malted barley and 30% nonmalted spelt; and (c) using a mixture of malted barley and 30% pre-cooked maize.

Table 2. Descriptive statistics of the range of the molecular weight distribution (kDa) according to different mashing procedures

	Temperature								
	45°C			55°C			63°C		
Mashing procedure	Infusion	One-step decoction	Two-step decoction	Infusion	One-step decoction	Two-step decoction	Infusion 30 min	Infusion, 15 min	Infusion, 1 min
				Standard barley malt					
Minimum	1.87	1.51	1.81	2.24	1.84	1.99	3.13	3.64	5.35
Median	2.99	2.49	2.82	3.73	3.4	3.19	5.21	5.56	6.81
Maximum	7.13	7.89	7.44	8.485	8.725	7.2	10.63	9.31	15.74
Mean	4.85	5.84	5.36	7.07	7.03	6.52	8.17	8.66	9.97
Standard deviation	2.17	2.19	2.05	3.11	2.78	2.64	3.4	2.63	3.22
Lower 95% CI	2.366	1.89	2.222	2.943	2.618	2.573	4.177	4.608	5.896
Upper 95% CI	4.572	4.563	4.656	5.501	5.624	4.839	7.46	7.258	10.44
				Barley malt + nonmalted spelt					
Minimum	2.7	3.31	2.91	3.75	3.48	3.69	4.33	5.42	5.92
Median	4.18	5.31	4.92	6.17	6.57	5.78	7.3	8.16	9.46
Maximum	9.36	10.23	8.88	13.15	11.7	11.5	14.9	13.1	15.9
Mean	4.85	5.84	5.36	7.07	7.03	6.52	8.17	8.66	9.97
Standard deviation	2.17	2.19	2.05	3.11	2.78	2.64	3.4	2.63	3.22
Lower 95% CI	3.388	4.297	3.978	4.981	5.17	4.755	5.889	6.895	7.812
Upper 95% CI	6.314	7.383	6.745	9.172	8.908	8.309	10.46	10.44	12.14
				Malted barley + pre-cooked maize					
Minimum	1.81	1.91	2.29	2.48	1.84	1.99	2.83	3.44	4.61
Median	2.61	2.82	3.19	3.765	3.4	3.71	4.84	4.96	6.71
Maximum	5.69	5.44	6.26	6.62	6.0	7.2	9.7	8.1	13.93
Mean	3.04	3.17	3.51	4.00	3.56	4.02	5.3	5.32	7.59
Standard deviation	1.29	1.19	1.27	1.32	1.36	1.70	2.22	1.60	2.98
Lower 95% CI	2.175	2.375	2.656	3.115	2.651	2.877	3.811	4.25	5.591
Upper 95% CI	3.909	3.981	4.371	4.891	4.488	5.163	6.799	6.404	9.603

of those beers obtained at 45°C ($p < 0.001$ and $p < 0.05$ respectively). Also, in this case the reduction of rest time to 15 and 1 min increased significantly ($p < 0.001$ respectively) the range of the MWD of the beers compared with those observed at 63°C and standard rest time. There were no statistical differences between the range of MWD of those beers produced using one- or two-step decoction or infusion at an initial temperature of 45°C. Similarly, there were no differences between the range of MWD obtained using one- or two-step decoction or infusion at an initial temperature of 55°C.

Comparisons of the MWD of the different beers according to the type of mash mixture. Figure 6 shows the median of the MWD of the beers produced with distinct types of adjuncts according to the mashing procedure. Statistical analysis was carried out using the Friedman test with Dunn post tests as described above. It can be seen that the medians of the MWD of beers produced using nonmalted spelt as adjunct were significantly higher than those produced using only barley malt ($p < 0.001$) or barley malt + pre-cooked maize ($p < 0.001$), at the same initial temperatures (45, 55 and 63°C). As described above, there were no differences according to the mashing method (infusion or decoction at initial temperatures of 45 and 55°C). The median of the MWD increased significantly ($p < 0.001$) at 63°C, regardless of the type of mash mixture, with this difference more noticeable when the time rest was reduced to 15 or 1 min at 63°C.

Discussion

Brewing adjuncts constitute an important aspect of worldwide brewing, contributing to overall production costs and to the quality of the final beer. The results showed that experimental beers produced with malted barley of 41 KI exhibited a high apparent degree of fermentation (>80%) as well as molecular weight distributions similar to those reported for commercial beers *(10)*. However, the use of distinct adjuncts, as well as variations on the parameters of the mashing process, may affect the properties of the resulting beer.

Effect of the initial temperature

As described previously, the initial mashing temperature is a critical parameter determining structural properties of the beer components *(10,20)*. The results of this work confirmed previous findings indicating that the use of 45 or 55°C as initial mashing temperature favours intense degradation of the cell wall of starch granules and long-lasting enzyme activity, allowing better degradation of carbohydrates *(5,21)*, resulting in low ranges of MWD of the beers. In contrast, the use of an initial mashing temperature of 63°C, just over the gelatinization temperature (62.4°C) of the starch granules of this malted barley, at which the activity of the β-amylases starts to decrease *(22)*, yielded beers with significant higher ranges of MWD. It has been recently reported that this effect influences sensorial properties, increasing the intensity of beer palate fullness *(10)*. The high significant increase on the range of the MWD observed when lowering the resting time to 1 or 15 min at an initial temperature of 63°C suggests that prolonging the β-amylase enzyme activity (longer rest times: 30–40 min) at this temperature, would yield beers with a better range of MWD. It is important to point out that, at an initial temperature of 63°C, using standard rest time (30–40 min), the MWD of the final beers increased significantly (compared with those obtained using initial temperatures of 45 and 55°C) without changing the ADF values. This resulted in beers with a strong palate fullness *(10)* without altering the standard recovery (ADF > 80%) during brewing.

Effect of the type of adjunct

It was observed that the use of pre-cooked maize as adjunct did not result in a noticeable change in the MWD of the resulting beers, compared with those obtained when using only malted barley. Conformation plots obtained by AF4/MALLS/RI showed a slightly more compact structure for the mixture of malted barley with pre-cooked maize, compared with that observed for malted barley alone. This is due to the high content of amylopectin in the maize starch granules *(23,24)*. Also, the previous cooking process of this maize meal (before marketing) may cause the breakdown of the crystalline structure of starch granules *(9)*, allowing direct access of amylases at the beginning of the mashing process. This may lead to a more efficient use of amylases, particularly of the β-amylase, the activity of which decreases rapidly at the gelatinization temperature of starch granules *(22)*. Therefore, the activity of amylases from malted barley was sufficient for the degradation of the high-molecular-weight carbohydrates of the mash mixture in fermentable sugars, resulting in ADF values similar to those obtained when using

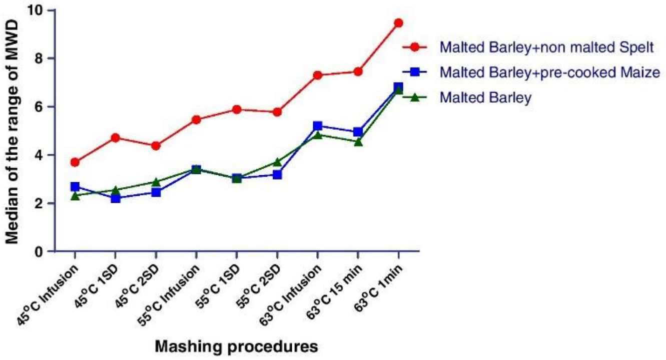


Figure 6. Median of the range of the molecular weight distribution (kDa) of experimental beers according to distinct mashing procedures.

malted barley alone. The content of the nitrogenous compounds found in this mixture was lower (around 30%) than when using malted barley or the mixture of malted barley with nonmalted spelt, which corresponded to the amount of pre-cooked meal (with low protein content). Moreover, it was also observed that the use of pre-cooked meal reduced lautering and filtration times, which is an advantage for use in high gravity brewing.

In contrast, the use of nonmalted cereal without addition of enzymes (nonmalted spelt in this case) resulted in significant lower values of ADF (<80%). AF4/MALLS/RI analysis of this mixture showed a less compact structure compared with the other mash mixtures, which may reflect a greater content of amylose (>35%) *(24)* compared with those reported for maize *(23,24)* and for barley *(25)*. Also, the presence of other carbohydrates, such as high-molecular-weight β-glucans in the nonmalted spelt, contributes to the more linear structure of this mixture. On the other hand, poor degradation of the cell wall of the starch granules of the nonmalted spelt *(26)* hinders the access of the enzymes to the starch granule during mashing. This would lower the proportion of degraded carbohydrates and proteins, and increase the dextrin and small peptide concentration *(27,28)* in the final beer. This was reflected by the higher MWD observed in these beers.

Effect of the type of mashing procedure

The choice of different mashing methods (infusion/decoction) may play an important role according to the type of the resulting beer. However, the possible influence on the quality of the beer is still not clear. For example, previous work has reported that the use of one-step decoction as a mashing procedure (using high-gravity brewing at industrial scale) yields wort with higher content of fermentable sugars compared with that obtained using a two-step decoction *(21)*. However, other studies comparing the mashing scheme (infusion/decoction) using sorghum as raw material have shown no differences in the maltose amounts in the wort extract, observing incomplete saccharification, probably owing to the quality of the raw material *(29)*. The results of this work show that the use of one- or two-step decoction or infusion (at a scale of 10 L) at similar initial temperatures (55 or 45°C) and standard rest time, resulted in beers exhibiting comparable ranges of MWD, as well as similar values of ADF. It must be considered that boiling of the cooked mash during decoction allows complete gelatinization of starch granules *(21,30)* facilitating the access of the amylases of the main mash into high molecular dextrins coming from the cooked mash. This effect may lead to comparable carbohydrate degradation processes during decoction or infusion, resulting in similar MWD of the final beers when using high quality raw materials at similar mashing conditions (initial temperature and rest time). Nevertheless, it should be mentioned that, in the industrial practice, initial temperatures are usually higher when using an infusion process compared with using a decoction process.

Taken together, the results of this work indicated that the range of the MWD of the resulting beers was tightly dependent on the quality of the raw material and the initial temperature of the mashing process, whereas that ADF values were affected only by the type of starch source mixture. Parameters such as the ADF give information about the amount of total degraded carbohydrates, while the MWD is an inherent property of carbohydrate structure. Thus, the MWD reflects mostly the carbohydrate composition in the final beer, which has been shown to influence sensorial properties, such as the intensity of palate fullness. The results of this work show that the AF4/MALLS/RI approach is adequate for measuring the ranges of MWD of beer components. Therefore, it could be an important tool for brewers for monitoring beer quality.

Acknowledgements

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Analytical Characterization of the Hydrolysis of Barley Malt Macromolecules During Enzymatic Degradation Over Time Using AF4/MALS/RI

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Abstract: The changes on the molecular weight distribution (MWD) and particle size distribution (PSD) during hydrolysis of barley malt in isothermal mashing procedures were determined using asymmetrical flow field flow fractionation coupled to multiangle laser light scattering and refractive index (AF4/MALS/RI). Mash/trials were focused on amylolytic starch degradation. Therefore, temperatures (65, 70, and 75 °C) were selected according to α- and β-amylases range of activity. Samples were produced by triplicate, tracking amylolytic processes over time periods from 10 to 90 min in each mash/trial. AF4/MALS/RI analysis demonstrated significant differences on the values of the MWD and PSD according to the temperature/time profile used. At mashing times over 30 min at a temperature of 65 °C, when α- and β-amylase are both active, the decrease over time of the MWD and PSD was significantly higher ($P < 0.005$) than at 70 °C when mainly α-amylase is active. At 75 °C, also the activity of α-amylase decreased and the MWD and PSD were significantly lower ($P < 0.005$) than at 70 or 65 °C at any time of the procedure. The MWD and PSD of beer components influence beer palate fullness, thus AF4/ MALS/RI would be a powerful tool for breweries to adapt their technological processes to obtain beers with particular sensorial attributes.

Keywords: barley malt, mashing procedures, MWD, PSD, AF4/MALS/RI, starch degradation

Practical Application: AF4/MALS/RI enabled the study of the changes of the MWD and the PSD due to the hydrolysis of barley malt during mashing processes. These parameters are associated to beer sensorial attributes, such as palate fullness and can be modified according to mashing temperature. Therefore, application of AF4/ MALS/RI would be a powerful approach helping to improve technological processes in breweries and to obtain different types of beers with particular sensorial attributes.

Introduction

Mashing as a dissolution process for substrate production for further fermentation is an essential step in beer production. Nowadays, proteolytic and cytolytic modifications are mainly done during malting, thereby state of the art of mashing procedures focuses on amylolytic starch degradation.

During brewing, degradation of starch, β-glucans, and proteins occurs by the activity of different enzymes including amylases, β-glucanases, and proteases. The activity of these enzymes depends mostly on temperature, pH, time, and wort concentration (Wunderlich and Back 2009). The optimal temperature for β-glucanases ranges from 40 to 45 °C, whereas that for proteases is between 45 and 50 °C. β-Amylase works well between 60 and 65 °C, α-amylase at 65 and 75 °C (Evans and others 2003; Guerra and others 2009), and Limit-dextrinase at 55 and 60 °C (Gous and Fox 2017). Gelatinization of barley starch, which is needed for amylases to degrade starch efficiently, occurs at 60 °C in the presence of α-amylases. The action of α-amylase leads to liquefaction of the gelatinized starch and further starch degradation takes place mostly by the joint action of α and β-amylases (Willaert 2007).

The temperature profile of mashing procedures and the duration of rest periods at specific temperatures determine alcohol content as well as the concentration of unfermented sugars, dextrins, and small peptides in the final beer (Narziß and others 2017). Unfermented sugars contribute to the sweetness, whereas dextrins have been associated to the palate fullness (Langstaff and Lewis 1993).

Analysis of wort carbohydrate composition has been an important tool in brewing for the selection of raw materials, yeast strains, product development, and quality control. However, little is known about macromolecular characteristics of wort and beer components and its influence on beer sensorial attributes such as bitterness, foam, and mouthfeel. More recently, application of asymmetrical field flow fractionation, which constitute a separation method suitable for large and polydisperse polysaccharides, due to its wide separation range and gentle conditions, has contributed to the analysis of macromolecular characteristics of beer components (Rübsam and others 2013a, 2013b; Dou and others 2015; Tügel and others 2015; Runyon 2016). For example, differences on the macromolecular composition of lager and porter beers could be determined using AF4/MALS/UV/dRI and were attributed to distinct technological parameters applied during brewing (Tügel and others 2015). Previous work, using AF4/MALS/dRI showed that physicochemical parameters such as the molecular weight distribution (MWD) of beer components can be modified according to mashing temperature, rest time, and type of adjunct in experimental beers (Rübsam and others 2013a). The use of an initial mashing temperature of 63 °C and standard rest time (30 to 40 min) yields beers with higher MWD compared with those obtained using lower initial temperatures of 45 and 55 °C (Rübsam and others 2013a), subsequently resulting in

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Table 1–Properties of the used barley malt according to MEBAK analytical procedures.

Variable (unit)	Values
Moisture content (%)	4.7
Friability (%)	84.1
Extract DBFG (%)	81.3
Diastatic power (Windisch-Kolbach)	243
Glassy corns (%)	1.6
Total protein (%)	10.4
Total soluble nitrogen (mg/100 g MDs)	694
Kolbach index (%)	41.2
Free amino-nitrogen (mg/100 g MDs)	141
Apparent attenuation limit (%)	82.5

beers with stronger palate fullness without altering the standard recovery (ADF>80%; Rübsam and others 2013b). This may happen through the modulation of amylases activity during mashing leading to the production of dextrins of different macromolecular structure (Rübsam and others 2013b), which are the main contributor to the palate fullness (Ragot and others 1989; Langstaff and Lewis 1993; Rübsam and others 2013b). Therefore, the aim of this work was to evaluate the feasibility of AF4/MALLS/RI to study the effect of temperature on the changes of the structure of barley malt macromolecules occurring due to starch hydrolysis during the mashing process.

In AF4, separation of molecules is carried out in a thin channel constituted by a solid impermeable upper wall and a porous permeable bottom wall (accumulation wall). Pumping the carrier liquid from the inlet along the channel generates a parabolic flow that moves the molecules of a sample towards the outlet. A cross-flow field is generated inside the channel acting on the molecules as they pass through. Thus, the molecules migrate through the channel according to their distance from the accumulation wall. Smaller particles located in the middle of the channel where the flow is faster eluted earlier whereas larger particles located closer to the accumulation wall and so eluted later. Hence, the molecules are separated due to their differences in translational diffusion coefficients. (Qureshi and Kok 2011; Yohannes and others 2011; Rübsam and others 2012; Nilsson 2013). The availability of different size pore membranes allows selectivity for the determination of a particular size range. AF4 can separate molecules with dimensions from 2 nm up to about 50 μm (Qureshi and Kok 2011; Yohannes and others 2011; Nilsson 2013). Connecting AF4 to MALS and RI detectors enables the determination of molar mass and root-mean-square radius of starch polymers as well as their distributions (Lee and others 2010; Rolland-Sabaté and others 2011; Juna and others 2011; Wahlund and others 2011; Juna and Huber 2012; Rühmkorf and others 2012; Shazia Jakob and others 2013; Zielke and others 2017).

Materials and Methods

Samples of barley malt with a Kolbach index (KI) of 41% (variety Marthe, Saaten Union, Isernhagen, Germany) were used for the mashing trials. The properties of the malt samples were determined according to MEBAK standard procedures (MEBAK 2002) and are described in Table 1.

Isotherm mashing procedures

Barley malt was processed in a laboratory mill (DLFU-Labormühle; Bühler, Braunschweig, Germany). The milling process was adjusted according to MEBAK procedure 3.1.4.2.1.

The isotherm mashing trials were carried out in a mash bath (Dinkelberg Analytics, Type BMW, Altmann Analytik GmbH&Co, Gablingen, Germany) at temperatures of 65, 70, and 75 °C, selected according to α- and β-amylase activity during mashing.

For each trial, 50 g of milled barley malt was mixed with 200 mL of tempered brew water (Narziß and others 2017) according to each isotherm in the mash bath. Samples (by triplicate) were obtained at different times (1, 10, 30, 60, 90 min) along the procedure in each mash/trial. They were cooled down to 10 °C and filtered (MN 514 ¼, Macherey-Nagel). Immediately 2 mL of each sample was analyzed using AF4/MALS/RI.

Determination of the MWD and particle size distribution of the mash samples

The weight molar mass (M_w) and root mean square (rms) radius of the mash samples were determined by means of asymmetrical flow field flow fractionation (AF4; Wyatt Technology Europe, Dernbach, Germany) coupled to multiangle laser light scattering (MALLS; Dawn, Heleos II, Wyatt Technology) and refractive index (RI; Agilent Series 1200 G1362A, Agilent Technologies, Waldbronn, Germany) as the quantitative detector, as described previously (Rübsam and others 2013a).

In these trials, the inserted spacer had a height of 350 μm, and a width of 21.5 mm at the widest point, approximately where the sample is focused (360 s). The length of the channel was of 240 mm. Fifty microliters of the samples were injected into the channel. Measurements were carried out at 25 °C. Carrier solvent composition was of sodium nitrate (50 nM) and 250 mg/L sodium azide in Milli-Q water. A 5-kDa membrane (Nadir regenerated cellulose, Wyatt Technology) was used for standards and mash samples. Inject flow was of 0.2 mL /min. Elution flow was 1 mL/min. The cross flow was diminished from 3 mL/ min to 0.1 mL/ min within 30 min and then maintained constant for 20 min at 0.1 mL/min and finally reduced to zero. Eighteen different scattering angles (10° to 160°) were used simultaneously to measure the intensity of the scattered light. An RI detector (dn/dc of 0.146 mL/g) was used to determine polysaccharides concentration in aqueous solvent. Because RI baselines are influenced by different salt and pressure conditions during an AF4 cross flow gradient, baselines of RI detector were subtracted by baselines from blank runs.

The values of the MWD and of the particle size distribution (PSD) were obtained using the Astra version 6.0.3.16 software (Wyatt Technology). They were expressed as the cumulative weight fraction as a function of the M_w and as the cumulative number fraction as a function of the rms radius, respectively.

Calibration of the AF4 procedures

Pullulan standards (1 g/L; PSS GmbH, Mainz, Germany) were used for the calibration of detectors (6, 10, 21.7, 48.8, 107, 200, 366, 708, and 2560 kDa). The ranges of the MWD of pullulan standards obtained by AF4/MALS/RI were compared to those provided by the manufacturer (Table 2). The differences of the values obtained by AF4/MALS/RI with those provided by the manufacturer were less than 10% in all the samples tested.

Statistical analysis

Variations of the range of MWD and PSD, among the mash/trials, were compared using Kruskal–Wallis Test with multiple Dunn's posttest (Daniel 1990) with a significance level (α) of

Table 2–Molecular weight of standard solutions.

Manufacturer molar mass M_w (g/mol)	AF4 obtained molar mass M_w (g/mol)	Error (%)
5900×10^3	6470×10^3	8.9
1000×10^4	9895×10^3	1.1
2170×10^4	2246×10^4	3.5
4880×10^4	4811×10^4	1.4
1070×10^5	1059×10^5	1.0
2000×10^5	1900×10^5	5.0
3660×10^5	3461×10^5	5.4
7080×10^5	7262×10^5	2.6
2560×10^6	22380×10^6	7.0

0.05 using (Graph Pad InStat version 3.05, Graph Pad Software, San Diego, Calif., U.S.A.)

Results and Discussion

Combining AF4/MALS/RI as an analytical tool with small scale—isothermal mashing trials proved to be a suitable approach to track starch amylolysis during mashing, obtaining important information about the macromolecular structure of starch degradation products which can be applied in beer production.

It must be considered that mash trials were carried at temperatures of 65, 70, and 75 °C (over gelatinization temperature) in consequence only starch molecules were mostly degraded. Low quantities of proteins and small cell wall–derived polysaccharides would be present in the grist; however, their molecular conformation cannot be significantly modified and they are in such low quantities that their contribution to the overall MWD or PSD of wort macromolecules is minimal. Thereby, the results obtained by means of AF4/MALS/RI analysis corresponded mainly to the MWD and PSD distributions of starch degradation products.

It was observed that when the mashing process was carried out at 65 °C the values of the MWD (Figure 1A) and the PSD (Figure 1B) of the obtained samples decreased significantly ($P < 0.005$) and proportionally to time, resulting in macromolecules with very low MWD and PSD at the end of the procedure (90 min). These results indicate that extended starch degradation throughout the mashing procedure occurred due to the concerted action of amylolytic enzymes at this temperature (Evans and others 2005). The α-amylase breaks starch amylose and amylopectin chains into lower dextrins molecules increasing the availability of sites for hydrolysis by β-amylase, which in turn breaks units of maltose from the non-reducing end of large dextrins (Robyt 2009) rendering small macromolecules of low PSD and MWD. Also, little activity of

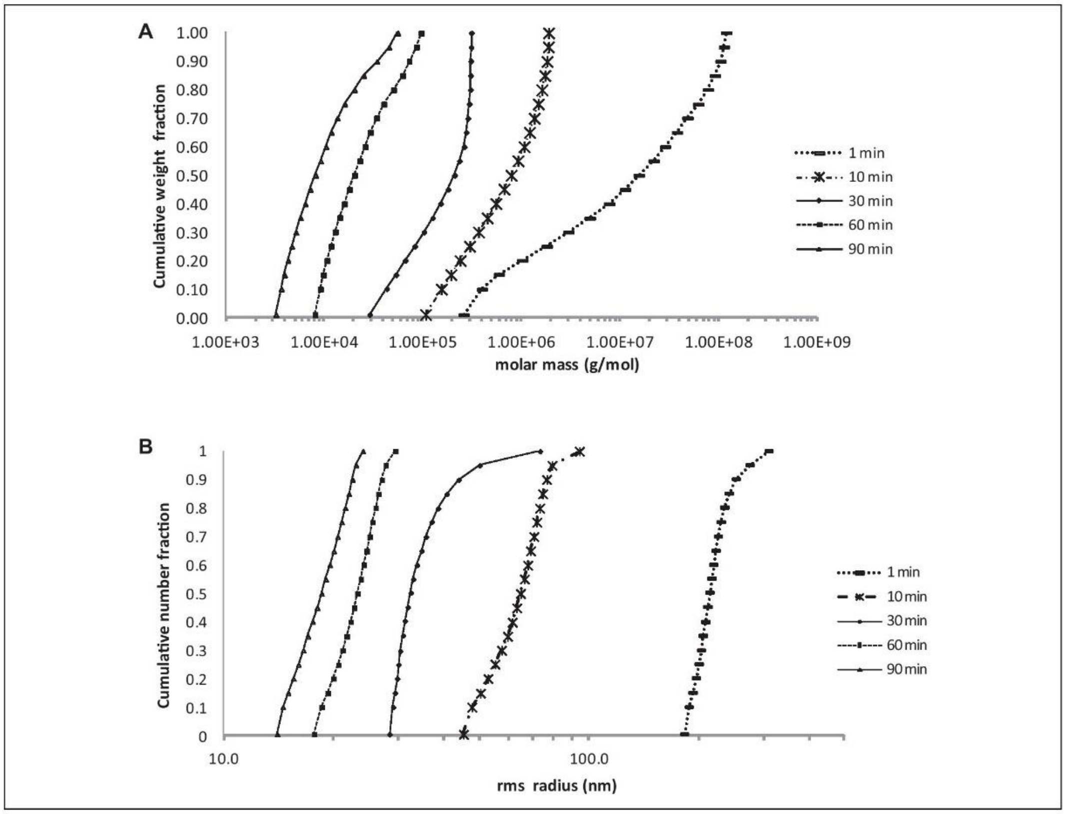

Figure 1–(A) Molecular weight distributions of the mash samples obtained at 65 °C. (B) Particle size distributions of the mash samples obtained at 65 °C.

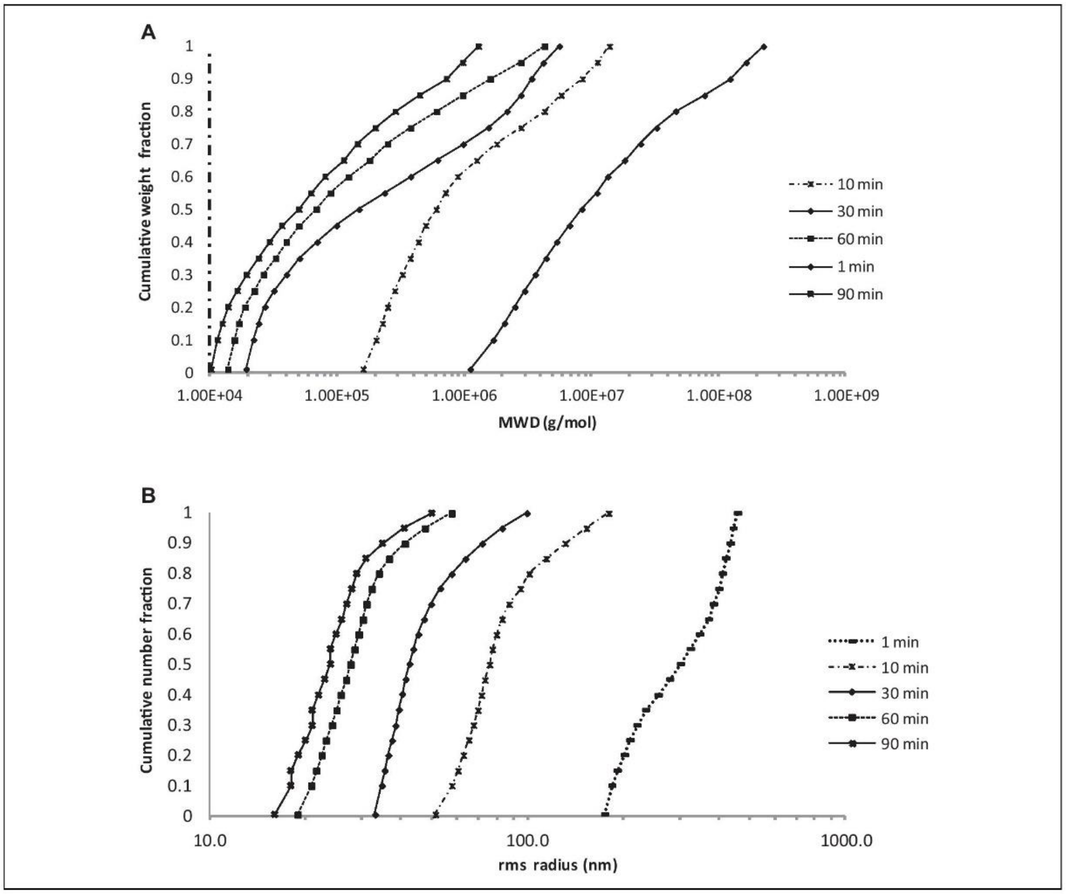

Figure 2–(A) Molecular weight distributions of the samples obtained at 70 °C. (B) Particle size distributions of the samples obtained at 70 °C.

Limit-dextrinase at this temperature has been reported (Gous and Fox 2017). This enzyme breaks α-1, 6 bonds at the branching points of the dextrins produced by the action of α-amylase, thus contributing to the production of small oligosaccharides with low MWD. The values of MWD and PSD of the obtained samples confirmed previous reports demonstrating that 65 °C is the optimal mashing temperature to obtain great amounts of fermentable sugars (Evans and others 2005; Duke and Henson 2011; Duke and others 2012; Hu and others 2014) and low quantities of small dextrins (Duke and Henson 2011; Rakete and Glomb 2013).

In contrast, when the mashing was conducted at a temperature of 70 °C, significant differences ($P < 0.005$) on the values of the MWD (Figure 2A) and PSD (Figure 2B; $P < 0.005$) were observed only at times ≤ 60 min. There were no significant differences between the values of MWD and PSD obtained at 60 min with those obtained at 90 min.

The decrease on the values of the MWD and PSD of starch degradation products obtained along the procedure can be attributed to the amylolytic activity reported at this mashing temperature. The activity of β-amylase at temperatures above 65 °C decrease rapidly, hence starch degradation mostly depended of the activity of α-amylase (Evans and others 2005; Duke an dothers 2012; Hu and others 2014) favoring the production of larger macromolecules with higher MWD particularly at times >30 min when the activity of α-amylase also slowly declines. Previous work have reported that rising the mashing temperature near 70 °C results in the production of greater amounts of maltodextrins of medium molar weight in the wort (Duke and Henson 2011). As one might expect, at times ≥ 30 min, degradation of starch was much slower at 70 °C than at 65 °C (Figure 4A and B) rendering macromolecules with higher MWD ($P < 0.005$) and PSD ($P < 0.005$).

The MWD and PSD of the samples obtained at 75 °C are shown in Figure 3A and B, respectively. There were no significant differences on the values of MWD of the samples obtained at times ≥ 30 min and the values of PSD decreased gradually from 10 to 60 min, being very similar between 60 and 90 min. Thus, a noticeable slowdown on starch degradation processes occurred when the mashing was extended beyond 60 min. This effect has been attributed to a significant drop of the activity of the α-amylase at

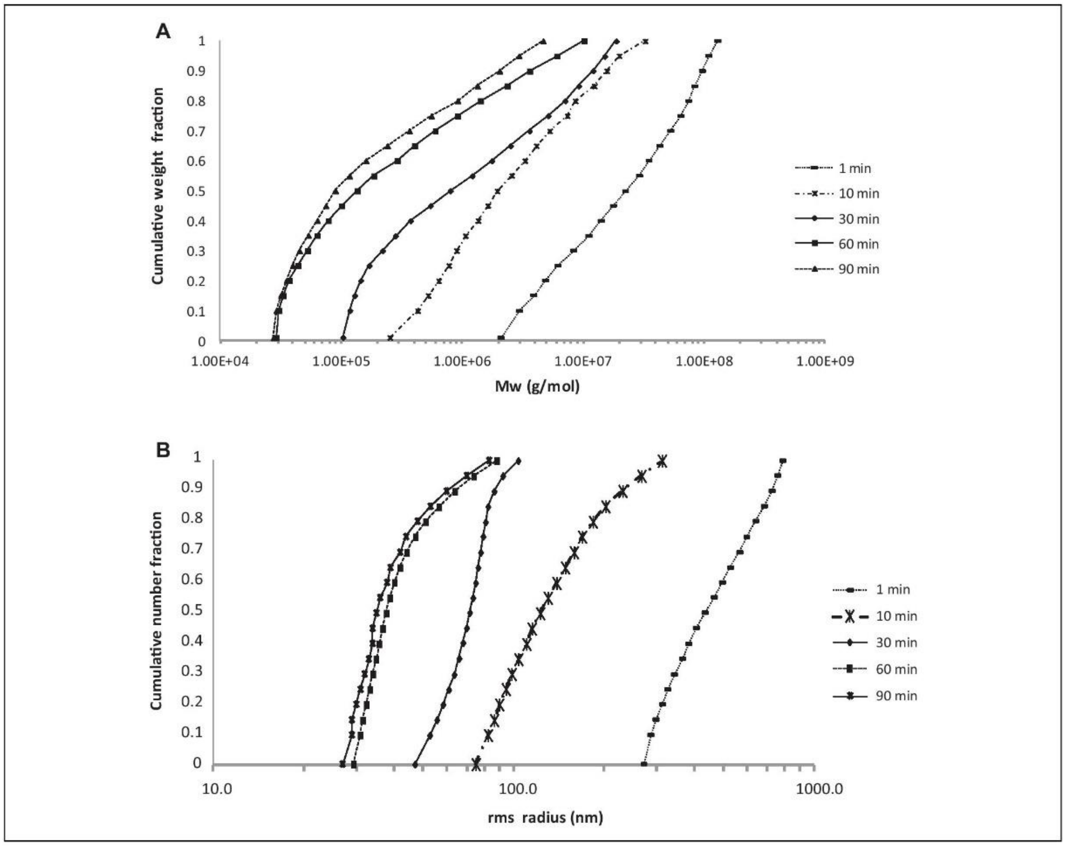

Figure 3–(A) Molecular weight distributions of the samples obtained at 75 °C. (B) Particle size distributions of the samples obtained at 75 °C.

temperatures higher than 70 °C (Evans and others 2005; Duke and Henson 2011; Duke and others 2012; Hu and others 2014). Accordingly, degradation processes were much slower than observed at 60 or 70 °C rendering macromolecules with significantly higher ($P < 0.005$) MWD and PSD at any time of the mashing procedure (Figure 4A and B). However, it has been reported that low levels of small oligosaccharides can be produced even at temperatures near 80 °C (Muller 2000).

Thereby, the selection of a particular temperature–time profile during mashing influences strongly the molecular structure of starch degradation products. These results support previous finding indicating that the mashing temperature is a key parameter determining the range of the MWD of the beer (Rübsam and others 2013a). However, understanding of the interactions between starch carbohydrate substrates with their hydrolyzing enzymes and their effect on the macromolecular characteristics of their products needs further research. Malt quality which is relevant in determining enzyme amylolytic activity (Duke and others 2012) has shown to influence the MWD of the final beer (Rübsam and others 2013a). Also mash thickness has been related to enzyme performance (Muller 2000). Thereby, several factors must be taken into account to adjust mashing conditions to obtain a particular macromolecular profile in the wort.

The definition of a particular MWD and PSD profile in the wort can be a relevant tool for the production of different types of beer with particular sensorial properties. The use of a mashing temperature of 65 °C, yielding small oligosaccharides with low MWD, would result in beers with lower palate fullness. In contrast, carrying out the mashing at a range temperature of 70 to 75 °C, which gives rise to dextrin molecules with high MWD, would lead to the production of beers with high palate fullness (Rübsam and others 2013b). Even more, it is well known that low alcohol-beers may have a sweet character due to the presence of high amounts of unfermented low molecular weight sugars. A high concentration of dextrins with high MWD in the wort may lower sweetness in the final beer, strengthening its palate fullness. Thus, selecting a mashing temperature ranging from 70 to 75 °C would be suitable, for example, for the production of alcohol free beers.

The importance of the determination of the MWD and PSD as well as other macromolecular characteristics during brewing (Tügel and others 2015) suggest strongly that application of AF4/MALS/RI would be a powerful tool for brewing. The use of this approach would guide breweries to adapt their technological processes in order to obtain different types of beers with particular sensorial attributes, such as the palate fullness, according to their own market.

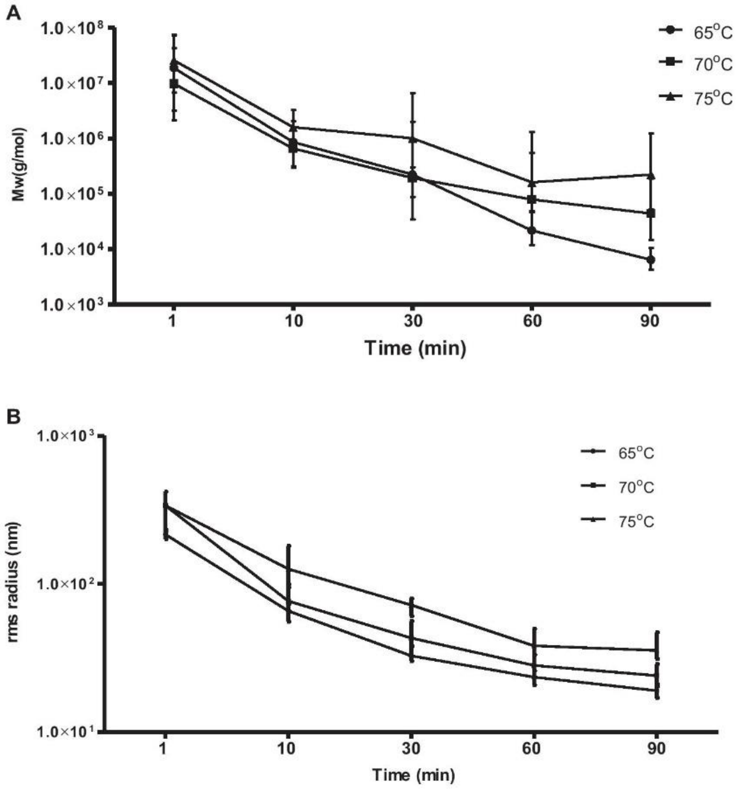

Figure 4–(A) Comparison of the decrease of the range of the MWD of the samples obtained at different isothermal trials. (B) Comparison of the decrease of the range of the PSD of the samples obtained at different isothermal trials.

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4 **Discussion**

The hydrolysis of starch to obtain fermentable sugars is the key process in brewing. The development of new techniques for the separation and characterization of starch polymers has allowed the incorporation of new parameters for the analysis and monitoring of brewing processes. Regardless of the purpose, separation of starch into its components is a complex process and the identification and characterization of the structural properties of the resulting macromolecules is still a challenge. Currently there is not a solely, best, technique. Therefore, the choice of an appropriated approach will depend of the objectives of a particular project.

SEC and AF4, as explained in chapter 1, have demonstrated to be suitable approaches to determine structural properties such as the molecular weight distribution and particle size distributions of large polymers. SEC has been useful for semi-quantitative comparisons between different starch macromolecules undergoing the process of digestion, however and because SEC separates by size instead of by molecular weight, the determination of quantitative size distribution of branched amylopectin molecules in a sample, without degradation cannot be accurately determined (Cave et al., 2009; Gidley et al., 2010). Other techniques such as high performance anion-exchange chromatography (HPAEC) have been used to obtain the chain length distribution of amylopectin after treatment with a debranching enzyme (Myers et al., 2008). Nevertheless this technique is restricted to relatively low degrees of polymerization.

On the other hand, Asymmetric Flow-Field Flow Fractionation (AF4) has emerged as a successful tool in the characterization of starch polymers as described in the introduction. Because AF4 relies on the diffusion of molecules and particles against a flow field into parabolic flow inside a channel and not on a stationary phase, shear forces during separation are small yielding good separations of polysaccharides. Diffusion coefficients (D) and so, the hydrodynamic radii (rh) can be obtained directly from elution times from the separation channel. The use of AF4 coupled with MALS and RI detectors allows direct molar mass determinations without calibration against molar mass standards. The availability of separation membranes with different pore sizes (1–30 kDa) allows a better selectivity for the determination of MWD and PSD. In addition AF4 provides the advantage to avoid clogging the column. Membranes can be easily replaced reducing costs of material. These characteristics highlighted AF4 as a potential approach for beer analysis including the monitoring of starch degradation processes during mashing as well as the determination of macromolecular characteristics of beer components.

Standardization of AF4 for beer analysis

For the purposes of this project, first AF4/MALS/RI experimental conditions were settled to determine the Molecular Weight Distributions of different commercial maltodextrins and commercial beers (Chapter 2) as well as of mash samples (Chapter 3, 4). The choice of the membrane and the validation of channel performance are important factors to take in account before starting a run. Nowadays, the availability of separation membranes with different pore sizes (1–30 kDa) allows good selectivity for the determination of MWD and PSD of particles in a wide range of molar masses and sizes (Rübsam et al. 2012). In this work a membrane of 1 KD was selected for the analysis of beers (Chapter 2) while a membrane of 5 KD was selected for the analysis of mash samples (Chapter 3, 4). The performance of the channel can be verified by injecting a small amount of a water soluble dye, sufficiently hydrophilic to not adsorb strongly at the membrane surface. In the case of cellulose based membranes a suitable dye is bromophenol blue (BPB) in aqueous solution at pH 6.5 while for hydrophilized polyethersulfone membranes an aqueous anilin blue solution is more suitable (Nilsson, 2013). The carrier liquid in AF4 is commonly aqueous varying depending on the analyte. Pure water is not advisable as any electrostatic interaction acting during separation may cause disturbances in the elution of sample components and poor reproducibility (Wahlund and Giddings 1987). Thereby a carrier liquid consisting of a dilute electrolyte: sodium nitrate (50 nM) and sodium azide (250 mg/L was used in this work. Optimization of the amount injected onto the channel was necessary to minimize overloading. A programmed cross flows decaying with time, was used instead of a constant cross flow because allows separation of polysaccharides over a wider size distribution and shorten time analysis (Chapter 2, 3, 4). The accuracy of the method was verified using pullulan standards of known molecular weights. The values of the molar weights of the standards provided by the manufactures did not varied significantly with those values obtained using AF4/MALS/RI (Chapter 2). Similar results were obtained in subsequent works (Chapter 3, 4) indicating the feasibility of the method to determine molecular weight distributions of these carbohydrates with great accuracy. Also a good reproducibility of the values of MWD was observed for different commercial maltodextrins, varying in a wide range of MWD (1.8–105 kDa). Larger elution volumes obtained by AF4 analysis corresponded to increasing ranges of MWD of the maltodextrins (Chapter 2), indicating that the application of AF4 technique was suitable for the separation of maltodextrins at this range of MWD.

Influence of the range of molecular weight distribution of beer components on the intensity of palate fullness

AF4 allowed the determination of the range of the MWD of beer components in samples of different commercial beers. Regardless of their similar alcohol and wort extract content, the range of the MWD varied significantly among the distinct commercial beers, ranging between 3 and 13 kDa (Chapter 2). Thus, as discussed below, differences on technological parameters of the brewing process, applied by the distinct breweries, would yield beers containing polymers differing in their MWD. Dextrins, which are the major components (75–80 %) of the residual unfermented solids present in beer (Ferreira, 1999) would be the main contributor to the overall MWD of beer components. However, other authors have reported that small sugars as well as nitrogenous compounds and β-glucan, in very low quantities also contribute to the MWD of the final beer (Tügel et al., 2015). The variations observed in the range of MWD of the components of the analyzed beers strongly reflected the differences in the intensity of the beer palate fullness perceived by the taster panel. Moreover significant correlations were found between the range of the MWD of the beer components and the rank of the intensity of the palate fullness. Thereby, AF4/MALS/RI would be a powerful tool to define a specific macromolecular beer profile associated to particular beer palate fullness.

Early studies have indicated that dextrins coming from starch degradation processes which are present in the final beer (20-30 g/L in a pilsner beer) influence the palate fullness (Langstaff and Lewis, 1993; Vermeylen, 1962). In this work, the influence of the

MWD of different maltodextrins (previously determined using AF4/MALS/RI) on the intensity of the palate fullness was studied by means of a spiking trial (beer + maltodextrin). The results showed that the effect of the maltodextrin concentration added to a pilsner beer on the intensity of the palate fullness was influenced by the range of MWD of the type of maltodextrin. Nevertheless, this effect could be appreciated only when intermediated concentrations of maltodextrin (5–20 g/L) were added to the beer samples. The addition of lower concentrations (2.5 g/L) of maltodextrin resulted in small differences in the perceived palate fullness. In contrast the addition of higher concentrations (40-80 g/L) strengthened the palate fullness so that the influence of the range of MWD of the maltodextrin could not be clearly appreciated. Moreover, the threshold concentration by which the palate fullness was perceived as different with respect to a control pilsner beer was found to be highly dependent on the range of MWD of the maltodextrin added. These results supports the concept that maltodextrins in beer increase the body and mouthfeel of the beer (Langstaff and Lewis, 1993; Liguori et al., 2015) and provide evidence indicating that this effect depends on the range of the MWD of the maltodextrins. It is important to point out that in this work, the values of the intensity of the palate fullness, obtained by the addition of maltodextrins of intermediated ranges of MWD in the spiking trial, corresponded to those observed for the commercial beer samples, suggesting that pilsner beers contain dextrins of intermediated MWD (3.4–22.3 kDa). However, further studies determining the MWD of maltodextrins in different types of beers are needed to confirm these results.

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Effect of the modification of technological parameters during mashing on the MWD of the produced beer and its association with the palate fullness

The effect of the initial temperature on the MWD of experimental beers produced in the laboratory was also investigated. The MWD of experimental beers was found to be strongly dependent on the initial mashing temperature (Chapter 2, 3). Nevertheless, this effect depended on the quality of the malted barley (Chapter 2). Thereby, using barley malt of 41 KI, significant higher ranges of MWD were obtained when the mashing process was initiated at 63°C compared to those obtained when the mashing process was initiated at 45°C or 55°C. As expected, these beers also exhibited stronger palate fullness. Using initial temperatures of 45°C or 55°C may favor a better degradation of proteins and β-glucans while partial degradation of starch by amylases may occur (Bamforth, 2009; Guerra et al., 2009). On the other hand, at 65°C just over gelatinization temperature the activity of the α-amylase decreases rapidly whereas the β-amylase activity is favored (Bamforth 2009; Guerra et al. 2009), leading to the production of dextrins with high molar masses which contribute to a higher range of molecular weight distribution of the total carbohydrates of the final beer. Also it must be considered that during malting, temperatures can reach up to 80°C (Briggs et al., 2004; Carvalho et al., 2016) leading to partial breaking of the crystalline structure of the starch granule (Greenwood and Milne, 1968; Robyt, 2009), allowing further slow degradation of starch at temperatures lower than gelatinization temperature, during the mashing process. In contrast, using barley malted with a 36 KI, with a low degree of modification, higher ranges of MWD were obtained when the mashing process was initiated at 55°C and 63°C compared to those obtained using a temperature of 45°C. In this case the cellular wall of the starch granule would not be degraded completely during the process of malting (Greenwood and Milne, 1968; Robyt, 2009), therefore hindering the access of the enzymes to the granule when the mashing is initiated at 55°C or 63°C. In contrast, at 45°C, the activity of proteases and glucanases intensively degrades the cellular wall of the granules during the mashing process (Greenwood and Milne 1968; Robyt 2009; Steiner, Gastl, and Becker 2011), allowing a better action of amylases, leading to the presence of carbohydrates of lower ranges of MWD in the final beer. Thereby, the use *of* AF4/MALS/RI allowed the determination of the influence of variations on technological parameters during mashing on the range of the MWD of the polymers present in the final beer which has shown to be an important analytical parameter for the evaluation of the beer palate fullness.

Despite the effect of the initial mashing temperature on the MWD of the beer it could also be demonstrated that lowering the rest times at a determined initial mashing temperature could modified the MWD of the resulting beers (Chapter 3). The significant increase on the range of the MWD observed when lowering the resting time to 1 or 15 min at an initial temperature of 63°C suggests that prolonging the β-amylase enzyme activity at this temperature (Evans et al. 2005), would yield beers with a better range of MWD. It is important to point out that, at an initial temperature of 63°C, using standard rest time (30–40 min), the MWD of the final beers increased significantly compared with those obtained using initial temperatures of 45°C and 55°C without changing the ADF values. Thereby, modifying technological parameters such as the rest time and temperature would lead to beers with different MWD without altering the standard recovery (ADF > 80%) during brewing.

Influence of the type of mashing procedure on the MWD of the final beer

The choice of different mashing methods (infusion/decoction) may play an important role according to the type of the resulting beer. However, the possible influence on the characteristics of the beer is still not clear. Previous work has reported that the use of one-step decoction as a mashing procedure (using high-gravity brewing at industrial scale) yields wort with higher content of fermentable sugars compared with that obtained using a two-step decoction (Montanari et al., 2005). However, the results of this work (Chapter 3) showed that the use of one- or two-step decoction or infusion (at a scale of 10 L) at similar initial temperatures (55 or 45°C) and standard rest time, using barley malt as raw material, resulted in beers exhibiting comparable ranges of MWD as well as similar values of ADF. Boiling of the cooked mash during decoction allows complete gelatinization of starch granules facilitating the access of the amylases of the main mash into high molecular dextrins coming from the cooked mash (Montanari et al., 2005). Thereby, the efficiency of carbohydrate degradation processes during decoction would be comparable to those occurring during infusion, resulting in similar MWD of the final beers when (Chapter 3). However, the range of MWD was higher ($p < 0.005$) when using infusion at an initial temperature of 63°C independently of the raw material used. In this regard it should be mentioned that, in the industrial practice, initial temperatures are usually higher when using an infusion process compared with those used in a decoction process.

Effect of different adjuncts on the MWD of the beer

It was observed that the use of pre-cooked maize as adjunct did not result in a significant change in the MWD of the resulting beers, compared with those obtained when using only malted barley (Chapter 3). Conformation plots obtained by AF4/MALLS/RI showed a slightly more compact structure for the macromolecules of the mixture of malted barley with pre-cooked maize, compared with that observed for the macromolecules of malted barley alone. This is due to the high content of amylopectin in the maize starch granules (Grandfeldt et al., 1993). Also, the previous cooking process of this maize meal may cause the breakdown of the crystalline structure of starch granules, allowing direct access of amylases at the beginning of the mashing process. This may lead to a more efficient use of amylases, particularly of the β-amylase, which activity decreases rapidly after gelatinization temperature of starch granules (Guerra et al., 2009). Therefore, the activity of amylases from malted barley was sufficient for the degradation of the high-molecular-weight carbohydrates of the mash mixture in fermentable sugars, resulting in ADF values similar to those obtained when using malted barley alone. The content of the nitrogenous compounds found in this mixture was lower (around 30%) than when using malted barley or the mixture of malted barley which corresponded to the amount of pre-cooked meal (with low protein content). Moreover, it was also observed that the use of pre-cooked meal reduced lautering and filtration times, which is an advantage for use in high gravity brewing.

In contrast, the use of non-malted cereal without addition of enzymes (nonmalted spelt in this case) resulted in significant lower values of ADF (<80%). In addition, AF4/MALLS/RI analysis showed that for the same range of molar weights the macromolecules of this mash mixture exhibited significant greater rms values compared with those observed for the macromolecular conformation of the other mixtures. These results suggest a more extended shape (less compact) of the macromolecules of this mixture which may reflect a greater content of amylose (>35%) (Tester and Morrison 1990) when compared with those reported for maize (Grandfeldt et al., 1993; Tester and Morrison, 1990) and for barley (Fox, 2008). Also, the presence of other carbohydrates, such as high-molecular-weight β-glucans in the non-malted spelt, contributes to the more linear structure of this mixture. On the other hand, poor degradation of the cell wall of the starch granules of the nonmalted spelt (Escarnot et al., 2012) hinders the access of the enzymes to the starch granule during mashing. Under these conditions carbohydrates and proteins would be less degraded yielding dextrins of higher MWD as well as a small concentration of protein derived molecules also contributing to the MWD of the final beer which together may increase the palate fullness.

Based on the obtained results, the overall MWD of beer components is a sensitive structural parameter that can be modified by different brewing conditions suggesting its usefulness as an indicator of beer characteristics.

Determination of the changes of MWD and PSD distributions of barley malt macromolecules that take place during starch amylolysis in mashing procedures using AF4/MALS/RI

AF4/MALS/RI was also used to monitor the changes on the MWD and PSD distributions of barley malt macromolecules that take place during enzymatic degradation in mashing procedures by means of isothermal mashing (Chapter 4). Mashing temperatures for the different trials were selected according to their effect on amylolytic enzymes (65°C, 70°C and 75°C). Thereby, only starch molecules were mostly degraded. Low quantities of proteins and small cell wall derived polysaccharides would be present in the grist, however, their molecular conformation cannot be significantly modified under these mashing conditions (65°C - 75°C) and they are in such low quantities that their contribution to the overall MWD or PSD of wort macromolecules is minimal. Based on the values of the MWD and PSD of the obtained samples, it was possible to confirm that variation of temperature and rest times influences amylases activity during mashing, supporting previous findings indicating that the mashing temperature is a key parameter determining the range of the MWD of the beer (Chapter 2, 3). Thereby, long rest times at 65°C may prolong the β-amylase activity at a temperature at which α-amylase is also active favoring extensive starch degradation (Evans et al. 2005) rendering small macromolecules of low PSD and MWD. Also some activity of limit-dextrinase enzyme at this temperature has been reported (Gous and Fox, 2017). This enzyme breaks α-1, 6 bonds at the branching points of the dextrins produced by the action of α-amylase, thus contributing to the production of small oligosaccharides with low MWD.

The values of MWD and PSD of the obtained samples confirmed previous reports demonstrating that 65°C is the optimal mashing temperature to obtain great amounts of fermentable sugars (Evans et al. 2005; Duke and Henson 2011; Duke, Vinje, and Henson 2012; Hu, Yu, et al. 2014; Rakete and Glomb 2013) and low quantities of small dextrins (Duke and Henson 2011; Rakete and Glomb 2013).

When the mashing was carried out at 70°C, starch degradation was mostly due to the action of α-amylase, resulting in extracts with higher molecular weight distributions and sizes compared to those obtained at 65°C, which may correspond to a higher content of branched limit dextrins. At 75°C, the activity of the α-amylase also decreased significantly (Evans et al. 2005; Hu, Yu, et al. 2014). Therefore, regardless of the mashing time, degradation of the malt samples was slower, rendering macromolecules with higher ranges of MWD and PSD (Chapter 4).

These results demonstrated that setting appropiated temperature and/or the rest time during mashing leads to particular MWD and PSD profiles in the wort, that can be characterized by means of AF4/MALS/RI indicating the relevance of this tool for the production of different types of beer with particular sensorial properties. The use of a mashing temperature of 65°C, yielding wort macromolecules with low MWD, would result in beers with low palate fullness. In contrast, carrying out the mashing at a range temperature of 70°C to 75°C, which gives rise to dextrin molecules with higher MWD, would lead to the production of beers with higher palate fullness (Chapter 4). Even more, it is well known that beers with low alcohol content may have a sweet character. A high concentration of dextrins with an elevated MWD in the wort may lower sweetness in the final beer, strengthening its palate fullness. Thus selecting a mashing temperature of 75°C would be suitable, for example, for the production of alcohol free beers (Chapter 4). However, understanding of the interactions between starch carbohydrate substrates with their hydrolyzing enzymes and their effect on the macromolecular characteristics of the resulting polymers present on the wort extract needs further research. For example, the Kolbach Index of the Barley Malt influenced the effect of the initial temperature on the MWD of the final beer (Chapter 3). On the other hand, mash thickness has been related to enzyme performance (Muller, 2000).

Thereby, several factors must be taken into account to adjust mashing conditions to obtain a particular macromolecular profile in the wort.

AF4/MALS/RI allowed with great accuracy the determination of the MWD and PSD distribution of the mash samples at any point of the mashing procedure. Also it was demonstrated that AF4/ MALS/RI can be routinely be used for the determination of the MWD distributions as a parameter to monitor the production of beers with a particular palate fullness. Thereby AF4 is a powerful tool for beer analysis guiding breweries to adapt their technological processes, facilitating manufacturing of different types of beers with particular sensorial attributes according to particular market specifications.

5 **Conclusions**:

The feasibility of AF4/MALS/RI to determine structural properties of beer components was demonstrated through the accuracy of the data obtained, confirming that this approach is appropriated to determine molecular weight distributions and sizes of complex carbohydrates. AF4/MALS/RI allowed the identification of the MWD of beer components from samples of different commercial beers which were found to be strongly associated to differences in the palate fullness among the distinct beers.

The results of this work indicated that the presence of maltodextrins of intermediated concentration influenced the palate fullness of the beer. This effect was highly dependent on the MWD of the maltodextrins, demonstrating that macromolecular characteristics of beer components are linked to sensory properties.

It was also demonstrated that mashing conditions such as the initial mashing temperature, rest times and the quality of barley malt and/or adjunts are critical parameters determining the overall MWD of beer components. Modifying technological parameters such as the rest time and temperature would lead to beers with different MWD without altering the standard recovery (ADF > 80%) during brewing while differences on malt quality can alter both the range of the MWD and ADF values of the produced beer.

Variations on the MWD and PSD distributions of barley malt macromolecules that take place during starch degradation in mashing procedures can be detected by means of AF4/MALS/RI. Variation of temperature and rest times influences amylases activity during mashing modifying MWD and sizes of the macromolecules present in the wort which may lead to beers with different sensorial attributes.

The results of this work constitute an important contribution to brewing studies. The determination of the MWD during brewing processes by means of AF4/MALS/RI could be a powerful tool guiding breweries to adapt their technological procedures to obtain different types of beers according to particular palate fullness.

Limitations of the study:

Despite the many advantages of the application of AF4/MALS/ RI for brewing analysis, the method has some limitations. Thus, when MALS is used as a detector, the light scattering data obtained enables the determination of the root mean square (rms) radius in a range greater than 10nm. To obtain information about the structural conformation of smaller particles, ranging from 1 to 10nm, the hydrodynamic radius (R_h) should be determined. For this purpose a Quasielastic Light Scattering (QELS) module can be coupled to AF4/MALS/RI. The combination of QELS and MALS allows simultaneous determination of absolute molar mass, root-mean-square radius from about 10–500 nm, and hydrodynamic radius from about 0.5–300 nm, helping the assessment of particle shape and conformation by comparing the hydrodynamic radius to either molecular weight or rms radius. Therefore, the use of AF4/ MALS/QELS/RI would give accurate information about the degree of polymerization of the molecules present in a mash sample or in the final beer.

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