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Published in:
Journal of the Institute of Brewing

DOI:
[10.1002/jib.195](https://doi.org/10.1002/jib.195)

2015

[Link to publication](#)

Citation for published version (APA):
Tugel, I., Runyon, R., Gomez, F., & Nilsson, L. (2015). Analysis of polysaccharide and proteinaceous macromolecules in beer using asymmetrical flow field-flow fractionation. *Journal of the Institute of Brewing*, 121(1), 44-48. <https://doi.org/10.1002/jib.195>

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Analysis of polysaccharide and proteinaceous macromolecules in beer using asymmetrical flow field-flow fractionation

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This paper demonstrates the potential of asymmetrical flow field flow fractionation coupled with online multi-angle light scattering, differential refractive index and UV detection for the fractionation and analysis of macromolecules in beer regarding their composition, molar mass (M) and relative concentration. The macromolecules in the liquid and foam of two types of beer, light lager and porter, were analysed in their native state with minimal sample preparation. The results showed the presence of three major populations of macromolecules. In lager beer liquid, the early eluting population has an average M of 2×10^4 g/mol and an intense UV absorbance at 280 nm suggesting the presence of proteinaceous macromolecules. The second and the third populations, which elute at consecutively longer retention times, have M ranging from 10^5 to 10^7 g/mol. They are not UV-active at 280 nm, suggesting the elution of polysaccharides. The second population was identified as β -glucans as a result of β -glucanase treatment. The third population was not identified in the present study. The results show that similar populations are present in lager beer foam and that the macromolecules appear to be present in a more aggregated state. The M range of macromolecules in porter beer liquid ranged from 10^5 to 10^8 g/mol. A fraction of macromolecules eluting at longer retention times is highly UV-active, which shows that there are great variations in the macromolecular profile of lager and porter beer. Copyright © 2015 The Institute of Brewing & Distilling

Keywords: beer; macromolecules; protein aggregation; polysaccharide; β -glucan; asymmetrical flow field-flow fractionation

Introduction

Beer contains a complex mixture of macromolecules, including proteins and polysaccharides. These macromolecules are derived as a result of the brewing process, which involves various modifications of proteins and cell wall polysaccharides of barley and other cereals (1). During brewing, the malting and mashing steps lead to partial enzymatic degradation of proteins into amino acids and polypeptides, which is followed by their aggregation and coagulation in the subsequent wort boiling, fermentation and maturation steps (2). Similarly, cell wall polysaccharides are enzymatically hydrolysed into smaller fragments during malting and mashing (3). The presence of some of the resulting proteinaceous molecules in beer is desired since they are known to contribute to foaming properties (4) and to mouth feel by influencing the palate fullness of beer (5). On the other hand, some of the proteinaceous molecules may need to be precipitated and removed from beer to prevent formation of haze (6). The extent of degradation in barley cell wall polysaccharides, particularly β -glucans and pentosans, plays a major role in both technological applications and the colloidal stability of beer (7). Insufficient hydrolysis and the presence of dissolved high molar mass (M) β -glucans (31×10^3 to 443×10^3 g/mol) increase the viscosity of wort and beer, which is known to cause filtration problems during brewing (8). Excessive amount of high- M β -glucans has been shown to cause turbidity in beer, which can be considered as a quality flaw (7). Consequently, being among the major quality determinants of beer, the analysis of macromolecules has found an important place in brewing research.

The heat-resistant foam-promoting beer protein fractions have been studied by conventional methods such as gel electrophoresis (9) and mass spectrometry (10). However, there remains a lack of information on the characterization of the foam-promoting beer proteins on a macromolecular level and how processes involved in brewing affect their integrity.

In the brewing industry the analysis of high- M β -glucans is mainly based on the methods described by the European Brewery Convention (EBC). The two most common approaches are fluorimetric and spectrophotometric methods, which are used to quantify high- M fractions of β -glucan. The fluorimetric method relies on the specific interaction of β -glucan ($M > 10^4$ g/mol) with the Calcofluor fluorochrome (11). One of the spectrophotometric methods is based on the enzymatic degradation of β -glucans into glucose units (11). This approach involves multiple sample preparation steps, including the precipitation of β -glucans. Another spectroscopic method relies on the ability of β -glucan of a specific size to form complexes with Congo red dye (12). This method requires size-dependent filtering as the sensitivity of the method depends greatly on the size of β -glucans. The recommended EBC methods are aimed at quantifying the high- M β -glucans, but do not provide information on the molecular weight

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distribution of β -glucans or focus on the degradation products of the other cell wall components. Residual cell wall polysaccharides in beer have been studied by high-performance liquid chromatography (HPLC); however no information on the M distribution of the macromolecules has been reported (13).

In this study, asymmetrical flow field-flow fractionation (AF4) coupled with online multi-angle light scattering (MALS), differential refractive index (dRI) and UV detection, was used to separate and investigate the M distribution of proteinaceous and polysaccharide macromolecules in beer. AF4 is a subclass of a family of separation methods called field-flow fractionation (14). In AF4, separation is based on differential diffusivity of different sample components under the influence of a cross-flow field as they flow through a thin ribbon-like open channel devoid of packing material. The absence of a stationary phase in AF4 enables the size-based separation of molecules with a wide range of M (10^3 to $>10^{10}$ g/mol) and sizes (2 nm to ~ 50 μ m). This maximizes sample recovery and allows for analysis to be achieved in a wide range of aqueous solutions (15). The relatively low pressures and shear forces help to preserve fragile aggregate structures and minimize shear-induced degradation of macromolecules (16). When coupled with suitable detectors, AF4 is a technique for determining the size and mass distribution, and other physicochemical properties of a variety of analytes such as polymers, proteins and colloidal particles (17,18). AF4 has already been shown to be a powerful characterization tool for food macromolecules (19). In brewing studies, AF4 has been previously used to determine the effects of starch source and mashing procedures on the overall molar mass distribution of beer (20). The purpose of this study was to investigate the feasibility of using AF4 coupled with online UV/MALS/dRI for beer analysis. AF4 allows for the analysis of beer macromolecules in their native state with minimal sample preparation, which means that samples that are taken at any point of the beer production line can be analysed with no requirement for additional sample preparation steps. Given the important role of residual macromolecules in determining the beer quality, the application of such advanced analytical methods in beer analysis is highly relevant and demanded.

Materials and methods

Beer samples

Two types of commercial beers, light lager (Carlsberg Export, Carlsberg Sweden AB) and porter (Carnegie Porter, Carlsberg Sweden AB), were investigated in the study. Beer samples were stored at room temperature in closed cans and bottles (as purchased) until the day of the experiments. All samples were investigated at minimum in duplicate.

Sample preparation

Liquid samples were taken directly from the bulk beer. Beer foam was formed by pouring the beer into a beaker. After 3 min, the foam was collected by a spoon and transferred into another beaker where it was left to collapse for 60 min at room temperature. A 2 mL aliquot from each sample was transferred into glass tube and placed in an ultrasonic bath for 30 min for degassing. The effect of sonication on beer macromolecules was investigated and no difference was observed in the AF4 fractograms or the M distribution.

Enzymatic treatment of beer samples

The β -glucan hydrolysis in the lager beer was carried out by adding 10 μ L β -glucanase/mL (BIOBETA P 100, Biocon) to the liquid and foam samples followed by incubation at 21 °C for 60 min. To ensure that changes did not occur owing to hemicellulase side activity of the β -glucanase used, lager beer liquid was treated with a xylanolytic enzyme, Depol 740 L (Biocatalysts), at the same concentration and under the same conditions as the β -glucanase treatment.

AF4 analysis equipment and separation parameters

The AF4 instrument (Wyatt Eclipse 3+, Wyatt Technology Europe, Germany) was coupled online with a MALS detector (Dawn Heleos II, Wyatt Technology, 658 nm wavelength), a dRI detector (Optilab T-REX, Wyatt Technology; 658 nm wavelength) and a UV detector (Jasco Corporation, Tokyo, Japan; 280 nm wavelength). The dRI detector was used as a non-selective concentration detector, whereas the UV detector enabled the selective detection of proteinaceous molecules. A Wyatt mini channel (Wyatt Technology Europe, Germany) equipped with a 350 μ m thick, wide spacer and a regenerated cellulose membrane with 10 kDa cut-off (Microdyn-Nadir GmbH, Wiesbaden, Germany) was used in the analysis.

The AF4 fractionation parameters were as follows: 200 μ L of sample was injected into the separation channel with an injection flow rate of 0.2 mL/min for 2 min under focusing flow conditions. Injection was followed by 3 min of additional focusing with a focus flow rate of 1 mL/min, after which sample elution began. The detector flow was kept constant at 1 mL/min during the entire analysis. The cross flow was kept constant at 2 mL/min in the first 10 min of elution and then exponentially decreased to 0.1 mL/min in 7 min with a half-life of 2.1 min. The exponential decay was followed by 26 min of elution under constant cross flow of 0.1 mL/min. The sample loop and channel were rinsed for 7 min by an elution/injection step with no cross flow at the end of each elution period. The carrier liquid was 200 mM phosphate buffer with 0.02% (w/v) NaN_3 added to prevent microbial growth. The pH of the carrier liquid was adjusted to 4.3 to approximate that of the beer. The output data was processed using Astra software, version 5.3.4.18 (Wyatt Technology). M was obtained using the Berry method (21,22) by fitting a straight line to data obtained at a 51.5–100.3° scattering angle. Specific refractive index (dn/dc) values of 0.185 and 0.146 mL/g were used in the calculation of M for the proteinaceous (populations 1) and non-proteinaceous (populations 2 and 3) sample components, respectively. As the actual dn/dc values are unknown, all values for M should be considered apparent. The second virial coefficient was assumed to be negligible. All samples were analysed under the same separation conditions.

Results and discussion

The AF4-UV/MALS/dRI method enabled the determination of M distribution and relative amount of beer macromolecules in their native state and with minimal sample preparation. Results obtained from the AF4 analyses are shown in Figs. 1–5.

Figure 1 overlays the MALS/RI/UV fractograms with the M distribution over the elution time of lager beer liquid. It can be seen that there are three distinct populations of macromolecules in the MALS-signal. The M range of the first population that elutes with

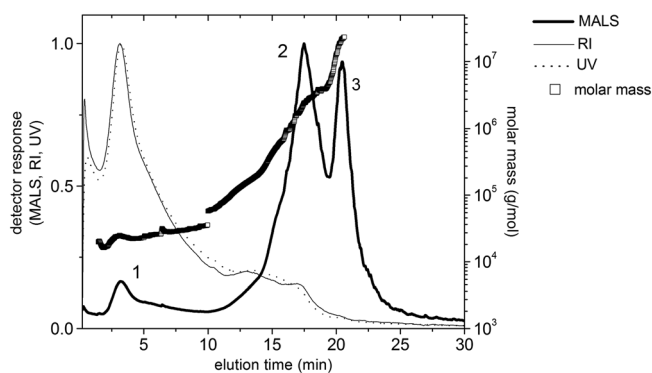


Figure 1. Elution profile and molar mass distribution for lager beer liquid. Numbers 1–3 indicate distinct populations of macromolecules.

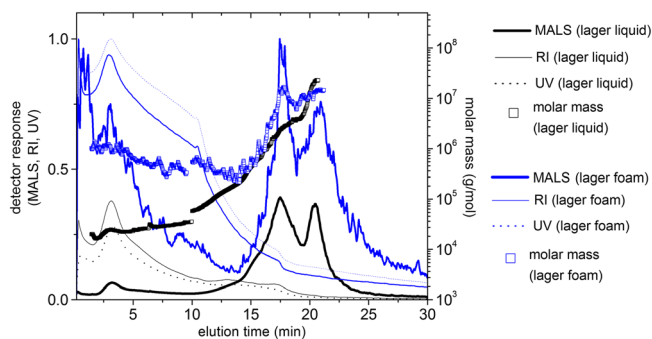


Figure 2. Elution profile and molar mass distribution for lager beer liquid and foam. This figure is available in colour online at wileyonlinelibrary.com/journal/brewing.

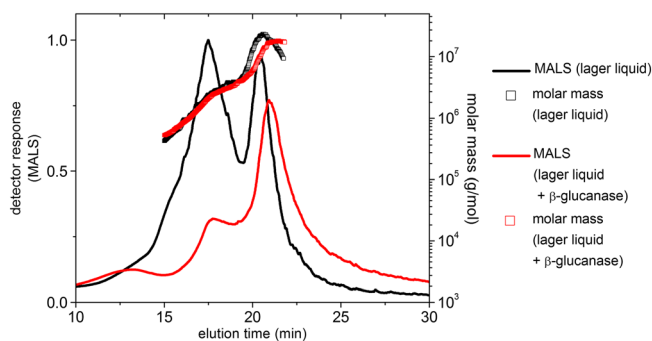


Figure 3. MALS-fractogram and molar mass distribution of lager beer liquid and lager beer liquid treated with β -glucanase. This figure is available in colour online at wileyonlinelibrary.com/journal/brewing.

a retention time (t_r) of 3 min and varies between approximately 1.6×10^4 and 2.4×10^4 g/mol. Population 1 is highly UV-active and has high dRI response. Populations 2 and 3 are in the late eluting region with retention times of 16 and 21 min, respectively. The M range of the second population, which has weak UV signal, is between 6.0×10^4 and 2.4×10^6 g/mol. Population 3 is not UV-active and it has an M range of 2.4×10^6 to 1.2×10^7 g/mol. It can be seen from the dRI signal in Fig. 1 that populations 2 and 3 are present at lower concentrations than population 1.

The results from the AF4 analysis of lager beer foam (Fig. 2) show the presence of the same three peaks pattern as in the lager beer liquid. The M range of population 1 for lager beer foam ranges between 6.1×10^5 and 7.4×10^5 g/mol. However, no conclusion can be drawn specifically about the M of population

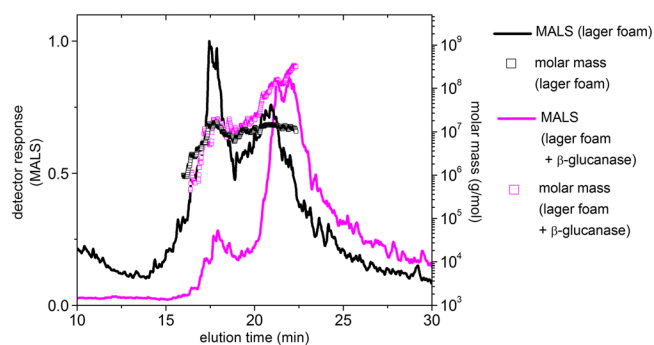


Figure 4. MALS-fractogram and molar mass distribution of lager beer foam and lager beer foam treated with β -glucanase. This figure is available in colour online at wileyonlinelibrary.com/journal/brewing.

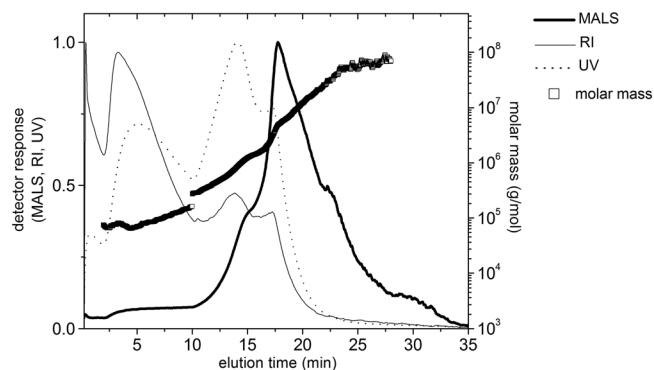


Figure 5. Elution profile and molar mass distribution for porter beer liquid.

1 in foam, as elution of low amounts of very large and small analytes appear to occur in parallel (23). This is reflected in Fig. 2 as a decrease in M with increasing elution time (1–14 min), that is, very small amounts of very large analytes co-eluting with the small analytes. Further method development would have to be undertaken to avoid this co-elution, which will be included in an upcoming study. The M range of populations 2 and 3 is between 7.7×10^5 and 1.1×10^7 g/mol. Similar to the lager beer liquid, the relative concentration of the macromolecules in population 1 is high compared with populations 2 and 3 in lager beer foam.

Beer contains a mixture of proteinaceous material derived from the proteolysis of barley proteins that go through the brewing process and, after various enzymatic and chemical modifications are found as glycoproteins as result of Maillard reactions (24). The M range of these polypeptides in beer varies between 5×10^3 and 1×10^5 g/mol (1). Hence, it is suggested that population 1 in lager beer liquid and foam contains proteinaceous material, which is also supported by the high UV signal in the corresponding region. The low UV signal of analytes eluting after 10 min shows that it is likely that populations 2 and 3 consist of polysaccharides. As shown in Fig. 2, the co-elution of macromolecules in population 1 in lager foam suggests that the macromolecules are more aggregated in the foam.

Figure 3 compares the MALS fractograms of the late eluting macromolecules over the elution time for lager beer liquid and lager beer liquid treated with β -glucanase. The addition of β -glucanase to lager beer liquid resulted in a decrease in the MALS-signal of population 2. Therefore, it is suggested that population 2 in lager beer liquid contains β -glucan and/or β -glucan

fractions with a range of M (13). In Fig. 1, it can be seen that population 2 has weak UV signal, which would not be expected if only β -glucans eluted in the population as they are not UV-active. Therefore, it is likely that β -glucans might be found to be closely associated with proteinaceous molecules. The effect of β -glucanase treatment on population 3 is not as significant as in population 2, but it is likely that it partly consists of β -glucans. It was suspected that population 3 contains other cell wall polysaccharides such as arabinoxylan, which has been reported to be present in beer in small amounts (13,25). However, the treatment with the xylanolytic enzyme had no effect on populations 2 and 3 (results not shown). The xylanolytic enzyme used has β -glucanase activity that is much lower in comparison to its xylanolytic activity, which is probably insufficient to have any effect under the applied conditions (26). This also suggested that the decrease in the MALS-signal of population 2 induced by the β -glucanase treatment was mostly due to the degradation of β -glucans, and not a result of the hemicellulose side activity of the enzyme used.

Figure 4 compares the MALS fractograms of the late eluting macromolecules of lager beer foam and lager beer foam treated with β -glucanase. The results obtained after treating lager beer foam with β -glucanase showed a decrease in the MALS-signal of population 2, suggesting the presence of β -glucans in beer foam. It is also seen in Figs. 2 and 4 that the AF4 separation in population 3 of lager beer foam is impaired, probably owing to co-elution of large aggregated species. The treatment with β -glucanase improved the separation, indicating that the enzyme influenced the structural properties of the aggregates, which suggests that β -glucans play a role in the formation of aggregates in beer foam. In addition, Figs. 3 and 4 show that the retention time was somewhat longer after enzyme treatment. This reflects an increase in the hydrodynamic size after enzyme treatment, which could be interpreted as the 'loosening up' of the aggregates, that is, an increase in hydrodynamic size. This shows that β -glucans might have a potential role in foam formation and stability, either by increasing viscosity at the air-liquid interface to retard liquid drainage (25) and/or by associating with proteins (27,28). It has been shown in previous reports that the level of non-starch polysaccharides is positively correlated with foam stability (25). Based on the current results, no conclusion can be drawn on the mechanism behind the contribution of macromolecules to the foaming properties of beer.

The MALS/UV/dRI fractograms of the porter liquid beer sample are overlaid in Fig. 5 along with the molar mass distribution. Comparing Fig. 5 with Fig. 1, it can be seen that under the same separation conditions the overall M range of macromolecules in the porter beer liquid was similar to lager beer liquid. However the elution profile was very different in appearance. For example, the UV absorbance of the macromolecules in the late eluting region was significantly different from the corresponding region in lager beer liquid. The macromolecules eluting between 12 to 20 min in porter liquid have a relatively high UV absorbance compared with lager liquid beer. The difference may be derived as a result of the difference in the malt source and/or different processing conditions applied during the malting and brewing of the two different beer types. A previous asymmetrical flow field-flow fractionation study on beer also demonstrated that the molar mass distribution of beer might vary based on the technological parameters applied during brewing (20).

The macromolecular profile of commercial beers might vary from batch to batch and depend on the brewing parameters

that are commercially non-disclosed, such as the malt source, processing temperatures and the incorporation of exogenous enzymes. The AF4-MALS/dRI/UV method used in this study demonstrates that differences in the macromolecular profile of different beer types and also in beer liquid and foam can be observed.

Conclusions

The results obtained in the reported study show that AF4-UV/MALS/dRI has great potential for the fractionation and analysis of macromolecules in beer regarding their composition with respect to being proteinaceous or non-proteinaceous, M and relative concentration. Using AF4-UV/MALS/dRI, it was possible to analyse beer components with minimal sample preparation and in their native state, which means that the influence of experimental conditions on sample integrity was minimized. The results showed that the tested commercial beers contain proteinaceous and polysaccharide macromolecules. It was possible to identify low amounts of high- M β -glucans in lager beer liquid and foam. It was observed that macromolecules were more aggregated in beer foam than in liquid beer. Differences were observed between the macromolecular profile of the investigated lager and porter beers and these are likely to result from the differences in malt source and brewing conditions. The results presented in this paper have shown that AF4-UV/MALS/dRI can be an important analytical method in brewing chemistry, and can be used for the further characterization of the complex macromolecular components in beer.

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