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# 2D-HPLC and MALDI-TOF/TOF analysis of barley proteins glycated during brewing

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ABSTRACT

The barley proteins have been the subject of interests of many research groups dealing with barley grains, malt and beer. The proteins which remain intact after harsh malting conditions influence the quality and flavor of beer. The characteristic feature of the proteins present in malt and beer is their extensive modification with carbohydrates, mainly glucose that comes from the starch degradation during technological processes. The degree of the protein glycation has an effect on the quality of malt and beer and on the properties of the beer foam. A combination of two-dimensional high performance liquid chromatography (2D-HPLC) and matrix assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF/TOF MS) was used for the analysis of the protein extracts that were reduced, alkylated, and degraded enzymatically without prior protein separation. This so-called "shot-gun" approach enabled us to determine glycation sites in one third of the proteins identified in the study and to propose potential glycation markers for fast and efficient monitoring during malting.

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## 1. Introduction

### 1.1. Barley proteins

Barley (*Hordeum vulgare*) is a plant commonly exploited in the brewing industry. In the course of the process of beer production, barley grains are germinated during malting to produce a large amount of α-amylase that degrades starch to sugars. The impact of the choice of a barley variety, and of the quality of grain and malt on beer properties is a major concern of the breweries worldwide. The most important components of the final product – beer – are proteins, carbohydrates, lipids, and acids from hops [1] that influence foam stability. Several proteins present in beer and foam have been characterized and found to be resistant to the malting processes [1]. The key beer and foam proteins are non-specific lipid transfer proteins (ns-LTP1 and ns-LTP2), protein Z, and hordeins that belong to the family of storage proteins. All these proteins are tolerant to high temperatures and resistant to proteolysis [2–5].

Starch degradation increases the amount of monosaccharides, mainly D-glucose, during malting in the malt. A combination of high concentration of monocarbohydrates, higher temperature, and long time of the process causes glycation of the proteins [6]. This non-enzymatic glycation is a result of side chain reactions, called Maillard reactions, between D-glucose and free ε-amino group in lysine and guanidino group in arginine [7]. D-Glucose reacts with free amino group giving a Schiff base that rapidly rearranges to form a more stable (1-deoxy-D-fructose-1-yl)-amino acid derivative called Amadori compound [8].

There were several attempts to develop relatively fast and simple method for protein identification and determination of glycation in malt and beer. So far, most of them considered an application of gel electrophoresis (GE), in-gel degradation, and mass spectrometry (MS) [9].

In this study, we present application of a modern analytical approach, known as "shot-gun" analysis, for characterization of barley proteins undergoing glycation during brewing.

The "shot-gun" experiment usually combines two-dimensional fractionation and mass spectrometry for protein digest analysis [10]. This approach is supposed to increase dynamic range and proteome coverage. The standard analysis involves an enzymatic degradation of a complex protein mixture. Resulted peptides are then separated by HPLC and eluted fractions are analyzed by mass spectrometry. Each isolated peptide should be fragmented, and the peptide sequence and protein identity is determined based on the fragmentation (MS/MS) spectra. The general goal in the "shot-gun" approach is determination of a huge number of proteins. In our case, we were mainly interested in detection and location of non-enzymatic glycations in barley malt.

*Abbreviations:* MALDI-TOF-MS, matrix assisted laser desorption/ionization time of flight mass spectrometry; 2D-HPLC, two-dimensional high performance liquid chromatography; ns-LTP, nonspecific lipid transfer protein; GE, gel electrophoresis; HCCA, α-cyano-4-hydroxycinnamic acid; ACN, acetonitril; *m*/*z*, mass to charge; TFA, trifluoroacetic acid.

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## 2. Materials and methods

### 2.1. Chemicals

All reagents (analytical grade) were obtained from Sigma (Schnelldorf, Germany). Chymotrypsin was obtained from Roche Diagnostics GmbH (Mannheim, Germany).

### 2.2. Samples

Barley seeds and malt (cultivar Jersey) were provided by Research Institute of Brewing and Malting, Brno, Czech Republic. Protein extracts were obtained by the method described below. Milled barley grain and malt (50 mg) were extracted twice in deionised water (500 μl) and centrifuged at 12,000 × *g*. The extraction was done at the room temperature in a shaker for 30 min. Supernatants from both extractions were collected and lyophilized at freeze dryer Alpha 1-2-LD plus, Christ, Hamburg, Germany (cycles: 1, freezing at −25 °C under atmospheric pressure; 2, main drying at −55 °C under vacuum, overnight).

The extracts were next dissolved in 200 μl of water and twice centrifuged in Nanosep 3.5K Omega tubes (Pall Corporation, Germany). The protein solution in the upper chamber was lyophilized overnight and the proteins were stored at −18 °C.

### 2.3. In-solution enzymatic degradation

The amount of 0.5 mg of each extract of barley grain and malt was incubated in 100 μl solution of 0.1 M dithiotreitol in 0.1 M ammonium bicarbonate for 1.5 h at 60 °C to reduce disulphide bridges. The reduced samples were dried in a speedvac and then 50 μl of 0.4 M iodoacetamide was added to alkylate thiol groups. The alkylation was carried out for 1 h at 50 °C in dark. The protein mixture was transferred to the Nanosep 3.5K Omega tubes and centrifuged at 3250 × *g* for 10 min. The procedure was repeated after adding 200 μl of water and the protein solutions were dried in a speedvac. The enzymatic degradation was carried out with 15 μl solution of enzyme chymotrypsin which was added to the alkylated protein extracts in ratio 1:50 (w:w). The enzymatic degradation with chymotrypsin was carried out overnight at 37 °C. The digested sample was desalted with Zip Tips C18 (OMIX Varian, 100 μl) using the procedure of the manufacturer and dried.

### 2.4. 2D-HPLC analysis

Peptides separation was carried out using 2D-HPLC Ultimate System 3000 (Dionex) equipped with a strong cation exchange column (SCX 10S, 300 μm I.D., 15 cm length, Dionex) and the nanocolumn RP C18 (PepMap 100, 75 μm I.D., 15 cm length, Dionex). A volume of 10 μl of protein digest was injected onto the SCX column, followed by elution with salt plug injections (2–2000 mM sodium chloride). The fractions resulting from salt plug injections onto the SCX column and the flow-through fraction were desalted on the "trapping" column, and subsequently loaded onto the RP C18 nano-LC column, where fractions were separated with a linear gradient of acetonitrile (ACN) in 0.04% TFA. The eluting peptides were mixed with the matrix solution (6 mg/ml CHCA in 66% ACN) and fractions were deposited every 30 s directly on a MALDI plate using ProBot TM Micro Fraction Collector (Dionex).

### 2.5. Mass spectrometry

All spectra were acquired on Applied Biosystems 4700 Proteomics Analyzer (Applied Biosystems, Framingham, MA) equipped with an Nd:YAG laser (355 nm) of 3–7 ns pulse and 200 Hz firing rate. The TOF/TOF analyzer operated at 200 Hz repetition rate in both MS and MS/MS modes. Data generated from MALDI-TOF/TOF MS instrument was analyzed using 4000 Data Explorer 3.6 software (Applied Biosystems, Framingham, MA, USA) and the automated Matrix Science MASCOT server. Protein identifications were assigned using the MASCOT search engine [11] using the NCBInr database.

## 3. Results and discussion

Monitoring of protein glycation during brewing was performed by comparing samples of barley seeds and grains. The choice of malt was based on a previous study of glycation of a selected protein (ns-LTP1) in barley malt, spent grains, sweet wort, boiled wort, green beer and finished beer [12]. The study revealed large amount of glycated protein in malt (presumably due to the relatively high temperature during malting and the abundance of glucose and maltose in malt), which was not altered by the malting process.

Water-soluble proteins were extracted from barley seeds and malt, lyophilized, concentrated and desalted by centrifugal ultrafiltration, and freeze-dried. The masses of dried malt extracts were approximately twice larger than those of the grain extracts. The extracts were analyzed by mass spectrometer to identify the proteins and their modifications. MS spectra of the proteins extracted from malt proved that the proteins were modified but remained uncleaved during the malting process. There were also signals at $m/z < 7$ kDa in the mass spectra. They may indicate presence of small peptides, but it is also possible that some percentage of the proteins was partially degraded. The sample of grain proteins was dominated by protein signals that corresponded to ns-LTP1 and ns-LTP2. Fig. 1A shows a mass spectrum of intact proteins extracted from barley seeds. No sign of glycations was observed in the spectrum. The mass spectrum of intact proteins from malt (Fig. 1B) revealed that these proteins were modified with hexoses, giving a typical ladder of signals differing by 162 Da [12]. Signals at $m/z$ equal to 7109, 9689, 9985 and 10279 Da correspond to ns-LTP2, ns-LTP1, ns-LTP1b, and ns-LTP1c [12]. The most intense ns-LTP1 signal in the malt spectrum was 9689 Da, indicating loss of the oxylipin modification of LTP1b (9985) during malting [12,9,13]. Identity of ns-LTP1 and ns-LTP2 was proved by MS/MS sequencing of proteins purified by reversed phase chromatography on a C18 column (gradient of 20–80% ACN/0.1% TFA in 25 min). Intense signals coming from unmodified proteins were also present in the spectrum. This demonstrates that non-enzymatic glycation does not lead to complete modification of the proteins during malting. The above mentioned higher overall amount of protein extract from malt rather than barley grain can be attributed to release of storage proteins during the germination process to a soluble form, along with the action of proteases produced during germination.

The crude protein extracts were alkylated and digested with chymotrypsin. The peptide mixtures were desalted and separated by 2D-HPLC (Fig. 2). The separation was carried out in the salt-plug mode. We monitored separation of the fractions eluted from the SCX column with 0, 2, 100, and 2000 mM sodium chloride solutions.

Enzymatic digestions of both samples resulted in very complex chromatographic profiles. There were differences between chromatograms of the protein digests of barley grains and malt. Intensities of some peptide signals differed and additional peaks were observed in the malt chromatograms. This indicated changes in the peptide composition in the eluted fractions reflecting differences in the protein quality and quantity in the original samples (Fig. 2). Among other contributions to the differences in chromatograms such as changes in the degree of enzymatic degradation during malting, the presence of glycated peptides was of our major interest.

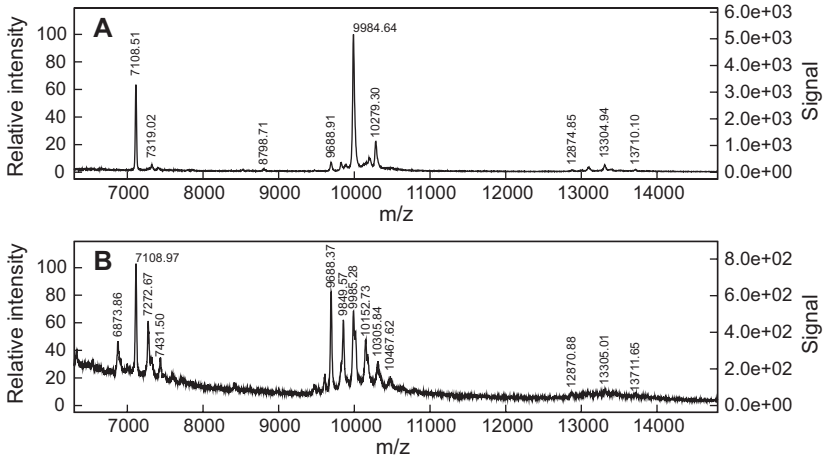


**Fig. 1.** MALDI-TOF mass spectra of extracts from barley seeds (A) and malt (B).

The focus on the glycations explains why chymotrypsin was used when preparing the protein digests. While the typical enzyme for proteomic study is trypsin, protein glycations occurring mostly at lysine and N-terminal residues lead to a choice of another proteolytic enzyme. Glycation of residues recognized by trypsin would lead to formation of relatively long peptides more difficult to be fragmented by MS/MS. Based on positive experience with a cleavage of glycated proteins in our previous study [14], chymotrypsin was chosen as the proteolytic enzyme.

Following the 2D-HPLC separation, the MALDI-TOF mass spectrometry analysis was applied in order to identify and compare peptide signals in individual fractions. The automatic MS and MS/MS analysis was carried out for all collected peptide fractions. As the analysis of glycations was the major goal of this study, we did not fragment all peptides. Instead, we focused our attention on the chromatographic fractions that exhibited differences in signals in the chromatograms of the analyzed samples. As many of such signals were missed by the automatic selection, MS/MS analysis was also applied to manually chosen signals unique to the malt peptides, i.e., not observed in chromatograms of the grain samples. In several cases both forms of the studied peptides, unmodified and glycated one, were observed in the malt digest. We also checked the fragmentation spectra of the corresponding unmodified version of the peptides to verify the identity of the glycated peptides.

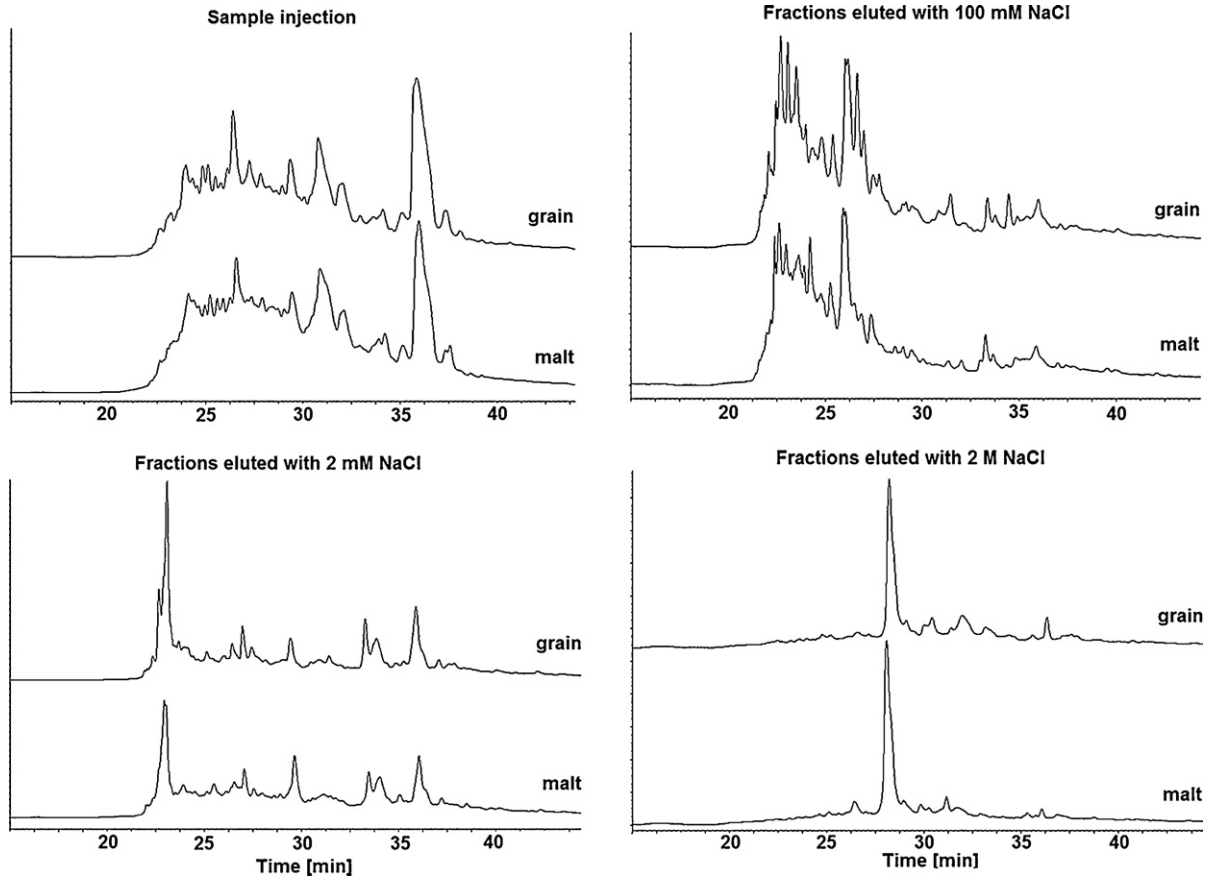


**Fig. 2.** Chromatograms of 2D separation of barley seeds and malt protein degraded with chymotrypsin.

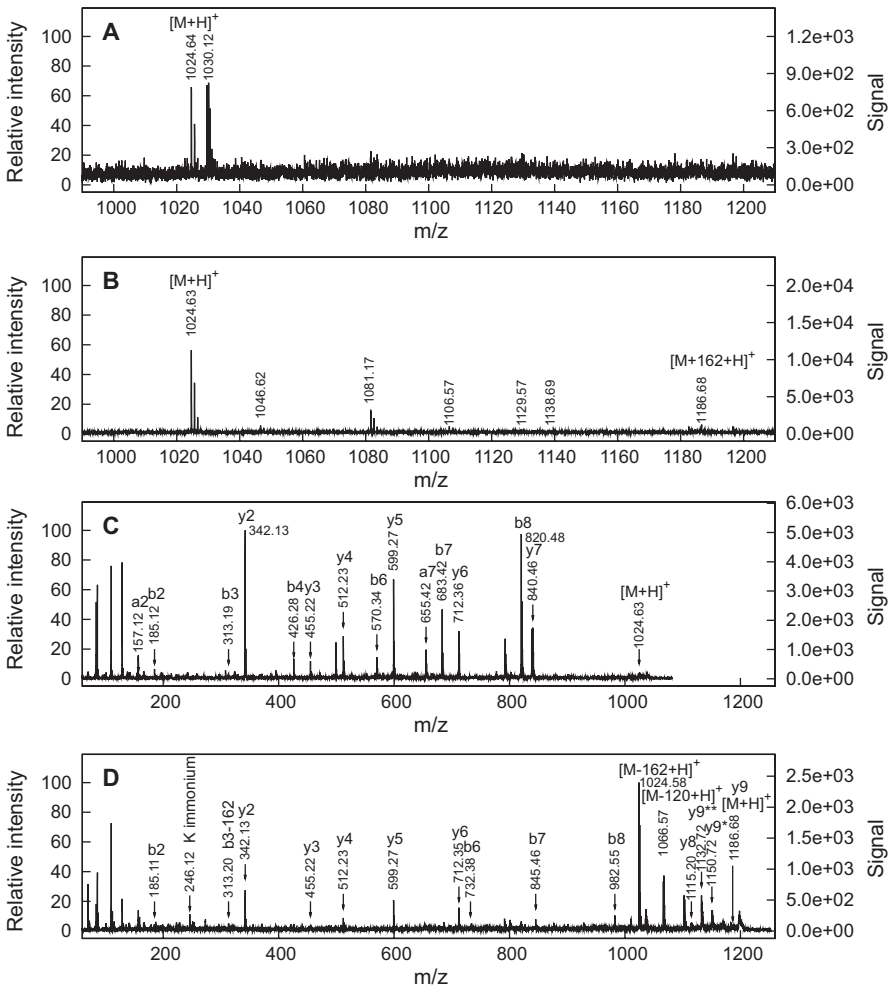


**Fig. 3.** MS (A and B) and MS/MS (C and D) spectra of beta-amylase AIKISGIHW. Spectra of peptides isolated from grain and malt are shown in panels A, C and B, D respectively. The observed fragments and the lysine immonium ions are indicated. Fragments with a loss of one and two water molecules are labeled with one and two asterisks, respectively.

Figs. 3 and 4 show representative MS and MS/MS spectra of studied samples that exhibited differences between corresponding fractions eluted from grain and malt protein digests after chymotryptic degradation. The spectrum in Fig. 3C and D is a result of fragmentation of a β-amylase peptide fragment (AI**K**ISGIHW) and the spectrum in Fig. 4C and D corresponds to a protein Z peptide (HIP**K**QTVEVGRF). The tandem mass spectra are dominated by the y-series of ions formed by breaking peptide bond and locating the charge at the C-terminal part of the peptide. Significant signals appear after a neutral loss of hexose $[M-162+H]^+$, of a $C_4H_8O_4$ $[M-120+H]^+$ fragment (Fig. 3B and D), and an immonium ion corresponding to glycation at the ε-amine group of lysine ($m/z = 246$). There was no signal at $m/z = 246$ in the fragmentation spectra of unmodified peptides, confirming its origin from a glycated lysine residue.

The complete list of the proteins identified in malt includes β-amylase with coverage 50%, C-hordein with coverage 57%, protein Z with coverage 31%, B1-hordein with coverage 39%, B hordein with coverage 31%, ns-LTP1 with coverage 68%, α-1,4-glycan-4-glucanohydrolase with coverage 45%, B3-hordein with coverage 25%, α-amylase/trypsin inhibitor CMa precursor with coverage 35%, D-hordein with coverage 40%, α-amylase with coverage 31%, cold-regulated protein with coverage 35%, β-glucosidase with coverage 33%, γ-hordein-3 with coverage 44%, pathogenesis-related protein 4 with coverage 30%, and glucose and ribitol dehydrogenase with coverage 28% (see Fig. S1 in Supplementary material).

Glycation of small proteins (ns-LTP1 and ns-LTP2) was detected already in the mass spectra of intact samples. The "shot-gun" approach based on enzymatic digestion provides two major improvements: detection of glycations is not limited by the size of the protein and a particular glycation site can be identified in the protein sequence. On the other hand, glycations of proteins present in small amounts in the samples may be difficult to detect in the digests due to the sensitivity limits, but may be visible in the spectra of intact proteins where glycation at different sites cumulatively contribute to the higher mass. ns-LTP2 may serve as an example – its low concentration resulted in a very poor peptide coverage and no glycation detected, but its intact MS clearly showed up to three hexose modifications per protein molecule. It is therefore advisable to combine both approaches. The "shot-gun" analysis detected glycation for five out of 16 proteins identified in this study (see Figs. 3D, 4D and Figs. S2–S6 in Supplementary materials). During the LC–MS analysis we were mostly interested in proteins that are crucial for malt and beer quality. The identified glycated proteins included ns-LTP1 and protein Z, characteristic proteins surviving brewing and thus influencing the quality of beer [15,16]. Therefore, ns-LTP1 and protein Z may serve as markers for monitoring glycation during the malting procedure influencing quality of the final product. The glycations of ns-LTP1 are clearly visible already in the intact MS spectrum of the malt extract (Fig. 1). Identification of glycations of four-times larger protein Z required the "shot-gun" approach, but the detected glycated peptide (HIPKQTVEVGRF)

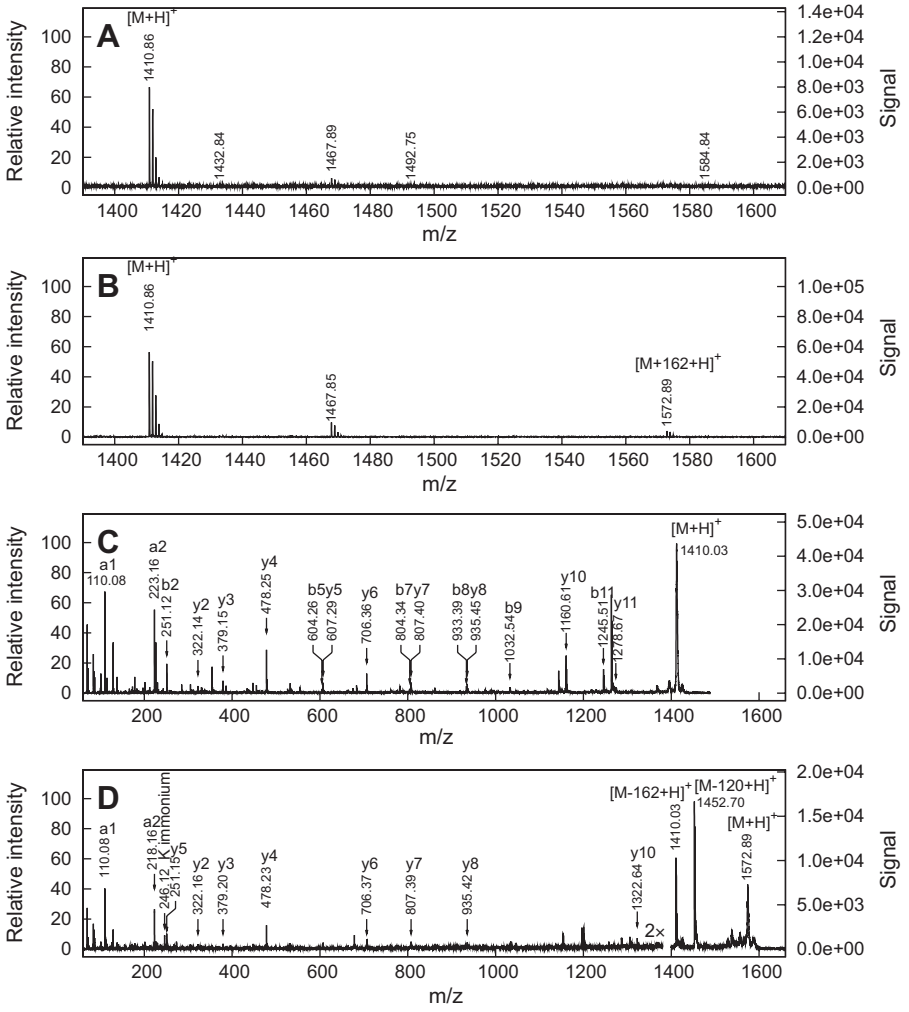


**Fig. 4.** MS (A and B) and MS/MS (C and D) spectra of protein Z peptide HIPKQTVEVGRF. Spectra of peptides isolated from grain and malt are shown in panels A, C and B, D respectively. The observed fragments and the lysine immonium ions are indicated. The region in a 2-fold magnification (labeled 2×) for the sake of clarity.

was found by automatic MS/MS analysis (Fig. 4D). Our results thus demonstrate that MALDI-TOF MS of intact extracts can be used as a quick method of monitoring glycations of the 10 kDa biomarker, ns-LTP1. A fully automated setup of the "shot-gun" analysis proved to be sufficient to observe glycation of the second beer quality marker, protein Z. The total experimental time of the 2D-HPLC separation and MS/MS screening was less than 24 h.

The presented method can be further improved by directly coupling the chromatographic separation to a suitable MS analyzer in an on-line LC–MS/MS manner with data-dependent $MS^n$ based on neutral losses for glycation, or immonium ion for glycated lysine. Such approach should allow identification of more glycation sites in addition to the most abundant targets described in this study.

## 4. Conclusions

The 2D salt chromatographic separation of digests of barley extracts reveals the complexity of the samples and provides a sufficient separation power to deal with these complex mixtures. It is especially important in the case when there are few peptides of the same molecular weight in the sample. Comparing the chromatograms of both grain and malt extracts enabled us to observe the differences introduced during malting and to determine fractions that may contain glycated peptides. This helped to focus the mass spectrometry analysis on these particular fraction intensities of which MS signals often required a manual selection for the MS/MS experiments. Combination of intact protein MS analysis and 2D LC–MS system proved to be an efficient tool for searching and analyzing potential biomarkers and theirs modifications.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2010.09.023.

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