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Development of brewing science in (and since) the late 19th century: molecular profiles of 110-130 year old beers

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Published in:
Food Chemistry

Link to article, DOI:
[10.1016/j.foodchem.2015.03.051](https://doi.org/10.1016/j.foodchem.2015.03.051)

Publication date:
2015

Document Version
Peer reviewed version

[Link back to DTU Orbit](#)

Citation (APA):

Walther, A., Ravasio, D., Qin, F., Wendland, J., & Meier, S. (2015). Development of brewing science in (and since) the late 19th century: molecular profiles of 110-130 year old beers. *Food Chemistry*, 183, 227-234. DOI: 10.1016/j.foodchem.2015.03.051

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17 **Abstract**

18

19 **The 19th century witnessed many advances in scientific enzymology and**
20 **microbiology that laid the foundations for modern biotechnological industries. In**
21 **the current study, we analyse the content of original lager beer samples from the**
22 **1880s, 1890s and 1900s with emphasis on the carbohydrate content and**
23 **composition. The historic samples include the oldest samples brewed with pure**
24 ***Saccharomyces carlsbergensis* yeast strains. While no detailed record of beer**
25 **pasteurization at the time is available, historic samples indicate a gradual**
26 **improvement of bottled beer handling from the 1880s to the 1900s, with**
27 **decreasing contamination by enzymatic and microbial activities over this time**
28 **span. Samples are sufficiently well preserved to allow comparisons to present-**
29 **day references, thus yielding molecular signatures of the effects of 20th century**
30 **science on beer production. Opposite to rather stable carbohydrate profiles,**
31 **some aldehydes reach up to 40-fold higher levels in the historic samples as**
32 **compared to present-day references.**

33

34

35 **1. Introduction**

36

37 The emergence of human societies is closely linked to the onset of agriculture, use of
38 controlled fireplaces, domestication of plants and craftsmanship for producing pottery
39 or metal kettles. These factors have been prerequisites for the production of beer and
40 other fermented beverages with analgesic, disinfectant and mind-altering effects
41 (McGovern, Zhang, Tang, Zhang, Hall, Moreau, et al., 2004; Meussdoerffer, 2009;
42 Walther, Hesselbart, & Wendland, 2014). In providing nutritive clean products,
43 whose consumption was a social act, fermented beverages themselves have
44 supposedly impacted strongly on the development of human societies (Libkind,
45 Hittinger, Valerio, Goncalves, Dover, Johnston, et al., 2011). The emergence of a
46 barley-based forerunner of modern beer dates at least to the time of 6000 BC. The
47 molecular archaeology of beer samples has recently attracted interest in the recovery
48 of a malt- and hop-containing beverage from a schooner that is believed to have sunk
49 in the 1840s in the Åland archipelago southwest of Finland (Wilhelmson,
50 Londesborouhg, & Juvonen, 2012). These samples with acidic pH and almost entirely
51 degraded maltooligosaccharides due to bacterial infections date back to the time
52 before the advent of pasteurization or pure brewing yeast cultures. Degraded samples
53 permit only limited conclusions about what kind of beer originally was filled into the
54 bottles.

55 Several advances in technology and knowledge mark the onset of scientific
56 brewing towards the end of the 19th century. Industrial-scale brewing had rapidly
57 emerged during the industrial revolution. In particular the popularity of cold-
58 fermented lager beers from the middle of the 19th century posed new challenges in the
59 cooling of brewing equipment and for yeast handling, however. Following the

60 development of scientific enzymology and microbiology, it became evident in 1860
61 that yeast fermentation converts sugar into ethanol and CO₂ (Sicard & Legras, 2011).
62 The problem of contamination by wild spoilage yeasts was aggravated by the
63 introduction of summertime lager beer fermentation. In 1883, Emil Christian Hansen
64 isolated pure cultures of *Saccharomyces* strains at the Carlsberg Laboratory. One of
65 these strains, Unterhefe No. 1, was chosen as production strain at the Carlsberg
66 brewery due to its convincing brewing performance, donated to other breweries and
67 entered the CBS strain collection as *Saccharomyces carlsbergensis* (Walther,
68 Hesselbart, & Wendland, 2014).

69 Samples dating back to the development of scientific brewing towards the end
70 of the 19th century could play a role in clarifying, how products and processes evolved
71 during this time and how advances in the brewing process have altered the final
72 product, lager beer, compared to its 19th century ancestors. In order to address these
73 questions, three beer bottles filled with original lager beer from the late 19th century
74 and early 20th century (Figure 1A) were obtained from the Carlsberg Museum bottle
75 collection. More specifically, the three bottles can be dated to the 1880s or 1890s for
76 bottle 1, the 1890s for bottle 2 and the 1900s for bottle 3. The extent of bottled beer
77 pasteurization at the time has not been determined. Yeast cells were found in the cell
78 slurries present in bottle 1, and Unterhefe No. 1 was isolated (Walther, Hesselbart, &
79 Wendland, 2014).

80 Here, the content of the bottles was subjected to detailed analysis with a
81 special emphasis on barley carbohydrates and aging markers. Complex changes in the
82 chemical composition of beer during storage constitute a well-known problem even
83 during normal shelf-life. Carbonyl compounds such as those deriving from Strecker
84 degradation, Maillard reactions and fatty acid oxidation are of particular importance

85 in beer staling (Rodrigues, Barros, Carvalho, Brandão, & Gil, 2011; Saison, De
86 Schutter, Delvaux, & Delvaux, 2008; Vanderhaegen, Neven, Verachtert, &
87 Derdelinckx, 2006). Despite of their volatility, acyclic, aromatic and heterocyclic
88 aldehyde markers formed by Strecker and Maillard reactions during beer aging were
89 detected in historic samples at levels up to 40-fold of a present-day reference sample
90 using gas chromatography.

91 Carbohydrates are the main carbon substrate of the fermentation process and
92 polysaccharide fragments in the product are informative on production conditions and
93 raw material usage (Marcone, Wang, Albabish, Nie, Somnarain, & Hill, 2013). Using
94 high-resolution nuclear magnetic resonance (NMR) spectroscopy, all samples were
95 found to contain significant quantities of carbohydrates, including fermentable sugars,
96 even after up to 130 years. Various unconventional carbohydrate signals that are less
97 abundant in present-day samples may be attributed to aging or to the use of 19th
98 century cereal varieties. Despite a limited sample set of unique historical bottles, the
99 carbohydrate profiles indicate a gradual improvement of beer handling from the 1880s
100 to the 1900s and provide a reference to visualize the improved usage of raw materials
101 due to 20th century biotechnology.

102

103 **2. Materials and methods**

104

105 **2.1 Samples**

106 Three dark brown glass bottles sealed with a cork and metal ring were obtained from
107 the Carlsberg Museum bottle collection filled with original lager beer from the late
108 19th century and early 20th century. The bottles were present as unique copies, and
109 were chosen as the oldest samples with entirely intact bottle glass as well as cork seals

110 and metal rings, thus indicating that the original material was contained in the bottles.
111 The times where the fillers named on the bottle labels were licensed for bottling were
112 used to date the bottles. All bottles were sprayed and washed extensively with ethanol
113 (70% v/v), opened in a clean bench and the bottle content was removed by pipetting
114 with sterile pipettes rather than by pouring. All three historic samples were
115 characterized with a DMA35 handheld density meter, while limitations in sample
116 amount allowed only beer sample 2 to be characterized with an Alcozyzer Beer
117 Analyzing System (Anton Paar, Graz, Austria). The liquid of the bottles was directly
118 frozen at -20 °C. The bottom slurry was used for the re-isolation of cells. For
119 comparison, a modern lager beer was used. Glucose, maltose, maltotriose,
120 maltotetraose, maltopentaose and maltohexaose reference compounds for HPLC were
121 obtained from Sigma-Aldrich (St. Louis, MO, USA), Dextra Laboratories (Reading,
122 UK) or Carbosynth (Compton, UK). Ethyl-glucoside was produced *in situ* as a
123 reference compound for alcoholic glucosides using glucose (20 mg/ml), ethanol (20
124 mg/ml) and 100 units of *Bacillus stearothermophilis* α -glucosidase (E.C. 3.2.1.20,
125 Megazyme, Bray, Ireland). The spectral positions of the ethyl- α -D-glucoside were
126 determined without product purification using ^1H - ^{13}C NMR spectroscopy.

127

128 **2.2 Spectroscopy**

129 NMR spectroscopy was applied as a robust, quantitative and non-destructive approach
130 for the detection of various organic molecule classes without the need for sample
131 derivatization or purification. Of the three historic and one reference (modern) lager
132 beer samples, 500 μl were mixed 1:1 with D_2O containing 20% (v/v) d_3 -acetic acid
133 (Sigma-Aldrich, St. Louis, MO, USA) in order to acquire 1D ^1H NMR spectra
134 including volatile compounds directly after the opening of the bottles (Nord, Vaag, &

135 Duus, 2004). The residual d₂-acetic acid signal of all samples was used for 1D ¹H
136 NMR spectral normalization. The 1D proton spectra of Figure 1B, C were acquired by
137 sampling 16384 complex data points during an acquisition time of 1.57 seconds and a
138 recycle delay of 10 seconds using excitation sculpting for water suppression, while
139 the 2D DQF-COSY spectrum was recorded with water suppression by presaturation
140 as a data matrix of 4096×512 complex data points sampling 0.85 and 0.11 seconds in
141 the direct and indirect dimensions, respectively. Ethanol content was measured by
142 integration relative to an alcohol free reference sample spiked with 4 mg/ml ethanol
143 and mixed 1:1 with D₂O containing 20% (v/v) d₃-acetic acid.

144 For ¹H-¹³C NMR spectroscopy, 1.5 ml of the beer samples were lyophilized
145 and redissolved in phosphate buffer (100 mM ,pH 6.5) in D₂O (99.9%; Cambridge
146 Isotope Laboratories, Andover, MA, USA). The strong buffer was used to stabilize
147 the pH and thus the signal positions of ionizable analytes. All NMR spectra were
148 recorded at 298 K on an 800 MHz Bruker (Fällanden, Switzerland) DRX
149 spectrometer equipped with a TCI CryoProbe and an 18.7 T magnet (Oxford Magnet
150 Technology, Oxford, UK) using conventional linear data sampling. ¹H-¹³C HSQC
151 spectra were recorded with a spectral sweep width of 30 ppm (6027.86 Hz) around a
152 ¹³C offset of 95.0 ppm. All spectra employed hard excitation and refocusing pulses,
153 only. Spectra were processed with extensive zero filling and shifted sine bell window
154 function in both dimensions. Data were processed in Topspin 2.1 (Bruker, Fällanden,
155 CH). All spectra were referenced relative to residual glucose with δ¹H₁=5.229 ppm
156 and δ¹³C₁=92.99 ppm for α-glucopyranose (Petersen, Hindsgaul, & Meier, 2014).

157

158 **2.3 Quantitation of unbranched starch fragments**

159 Fluorescence labeling with 2-aminobenzamide prior to hydrophobic interaction liquid
160 chromatography (HILIC) analysis on a UPLC (Waters) was performed on reference
161 standards and beer as follows (Bøjstrup, Petersen, Beeren, Hindsgaul, & Meier, 2013;
162 Hughes & Johnson, 1982). Glucose, maltose, maltotriose, maltotetraose,
163 maltopentaose and maltohexaose were dissolved in water to concentrations of 1
164 mg/ml. 100 µl of these solutions were lyophilized. 100 µl of each beer sample were
165 lyophilized. To these lyophilized samples was added 100 µl of a 1 M solution of 2-
166 aminobenzamide in DMSO/AcOH (7:3), followed by adding 100 µl of a 1 M solution
167 of NaBH₃CN in DMSO/AcOH (7:3). Samples were whirlmixed and incubated for 4
168 hours at 60 °C, then cooled to room temperature and diluted (1:400) with a mixture of
169 10 mM ammonium formate buffer (pH 4.5) and acetonitrile (22:78). Samples were
170 centrifuged and directly analyzed. Of the labeled samples, 5 µl were injected into
171 Waters Acquity UPLC System equipped with Acquity glycan column, FLR detector
172 (excitation wavelength of 350 nm and emission wavelength of 420 nm) using an
173 Acquity UPLC BEH glycan 1.7 µm, 2.1×150 mm column with a VanGuard BEH
174 glycan 1.7 µm, 2.1×5 mm pre-column at room temperature.

175

176 **2.4 Solid-phase microextraction and chromatography**

177 Aldehydes and other volatile compounds were extracted using solid-phase
178 microextraction prior to detection and quantification by gas chromatographic
179 separation coupled to mass spectrometric analysis (GC-MS) with a Thermo Scientific
180 Quantum GC triple quadrupole mass spectrometer. As an internal standard, 2-octanol
181 was added to each sample. Samples (2.5 ml) were prepared in 20 ml vials by adding
182 appropriate amounts of sodium chloride (final concentration 40 mg/ml), 50 µl NaN₃
183 (0.1 % w/v), 25 µl 2-octanol (final concentration of 200 µg/l) and ascorbic acid (to a

184 final concentration of 20 mg/ml). All samples were incubated for 10 min at 50 °C.
185 Solid-phase microextraction was performed using a
186 divinylbenzene/carboxen/polydimethylsiloxane fiber (DVB-CAR-PDMS) for an
187 extraction time of 40 min. A solgel-wax GC column (30 m/i.d. 0.25 mm/Film 0.25
188 µm) was used for all analyses. Helium gas was used as the carrier with a gas flow rate
189 of 1.2 ml/min. The thermal desorption time of analytes was 4 min. The MS detector
190 was operated in full scan mode at 70 eV with a scan range from 35 to 350 m/z. Data
191 were analysed using the ThermoXcalibur software (Version 2.2 SP1.48, Thermo
192 scientific) and compound identification based on the NIST version 2.0 mass spectral
193 database.

194

195 **3. Results**

196

197 **3.1 1D NMR spectra of old beer samples**

198 High-resolution nuclear magnetic resonance spectroscopy is an established method
199 for the compositional analysis of non-fractionated beer samples (Duarte, Barros,
200 Almeida, Spraul, & Gil, 2004; Duarte, Godejohann, Braumann, Spraul, & Gil, 2003;
201 D. Lachenmeier, Frank, Humpfer, Schäfer, Keller, Mörtter, et al., 2005). The complex
202 constitution of carbohydrates in beer has been addressed using NMR spectroscopy of
203 non-fractionated beer samples only rather recently (Beeren, Petersen, Bojstrup,
204 Hindsgaul, & Meier, 2013; Bøjstrup, Petersen, Beeren, Hindsgaul, & Meier, 2013;
205 Petersen, Hindsgaul, & Meier, 2014; Petersen, Motawie, Moller, Hindsgaul, & Meier,
206 2014; Petersen, Nilsson, Bojstrup, Hindsgaul, & Meier, 2014). Challenges in the
207 NMR analysis of carbohydrates relate to the narrow ¹H chemical shift range,
208 congestion with the water signal and the vast constitutional complexity (including

209 varying branching, ring sizes and anomeric configurations) of carbohydrates (Laine,
210 1994). Figure 1B displays ^1H NMR spectra of the historic beer samples designated
211 bottle 1, bottle 2 and bottle 3 (bottle 3 being the youngest sample at 110 years of age)
212 in comparison to a present-day lager beer. Carbohydrates are observed at considerable
213 amounts in all samples, whereas starch fragments (α -glucans) remain the main
214 constituents of samples above 100 years of age.

215 A noteworthy trend is observed for samples bottle 1, bottle 2 and bottle 3 in
216 the spectra of Figure 1B, where newer beers contain more $\alpha(1-4)$ -glucan signals (^1H
217 chemical shifts of 5.3-5.5 ppm), while older beers contain less $\alpha(1-4)$ -glucan signal
218 but more reducing end signals (^1H chemical shifts around 5.23 ppm). The spectral
219 signature of $\alpha(1-6)$ -branch points on the other hand is nearly identical in all historic
220 samples, but is different in present-day lager, which displays more extensive cleavage
221 of amylopectin towards the non-reducing end of branch points. While limit dextrans
222 appear to be stable in the historic samples, maltooligosaccharides apparently have
223 been degraded to smaller sugars, presumably after bottling, especially in the historic
224 beer bottle 1. Such activity in the bottled beer is consistent with the purification of
225 living Unterhefe yeast cells from the historic bottle 1. Specifically, the beer sample
226 from bottle 3 shows α -reducing end signals with an integral of 8% of the $\alpha(1-4)$ -
227 glucan signal and $\alpha(1-6)$ glucan signals with an integral of 14% of the $\alpha(1-4)$ -glucan
228 signals, as compared to 14% α -reducing end and 16% $\alpha(1-6)$ glucan signals for bottle
229 2, and 17% α -reducing end and 24% $\alpha(1-6)$ glucan signals for bottle 1 due to a loss of
230 intact $\alpha(1-4)$ -glycosidic bonds in the older bottles. As compared to a present day
231 reference with 12 % α -reducing end and 23% $\alpha(1-6)$ glucan signals relative to $\alpha(1-$
232 4)-glucan signals, bottle 3 contains significantly more intact $\alpha(1-4)$ -glycosidic bonds.

233 Clues as to the differential stability of historic beer samples were sought in the
234 aliphatic and aromatic spectral regions of ^1H NMR spectra (Figures 1C, D). The
235 ethanol contents were 2.9%, 4.8% and 2.8% for samples of bottles 1, 2 and 3,
236 respectively. Historic samples showed classical amino acid and organic acid profiles
237 of beers. All historic samples had higher organic acid content than a present-day
238 reference, particularly more formate, lactate and acetate. The pH values of samples
239 from bottles 1, 2 and 3 were 4.95, 4.4 and 4.3, respectively, and thus comparable to
240 present-day beers. In comparison, values for bacterially degraded historic samples
241 from the 1840s were as low as pH 3 (Wilhelmson, Londesborouhg, & Juvonen,
242 2012). The higher pH of the beer from bottle 1, above the pKa of acetate, could
243 explain the survival of yeast cells, supposedly in a dormant state, for 130 years in this
244 particular bottle. Yeast cells clearly are not dormant due to the lack of a fermentable
245 carbon source in bottle 1 (Figure 1B), but presumably due to the lack of other
246 nutrients. The apparent extract was determined with a handheld density meter to 1.8,
247 2.3 and 4.9% for samples of bottles 1, 2 and 3, respectively. In addition, the sample of
248 bottle 2 was characterized on an Alcoalyzer Beer Analyzing System to yield an EBC
249 color value of 54.8%, 3,59% real extract, 61,52 %real degree of fermentation and
250 2,3% apparent extract.

251

252 **3.2 Maltooligosaccharide quantitation**

253 The varying abundance and degradation of maltooligosaccharides in the beer samples
254 was probed by hydrophobic interaction liquid chromatography (HILIC) after the
255 fluorescence labeling of beer samples. Chromatograms of Figure 2 support the ^1H
256 NMR spectroscopic finding of maltooligosaccharide degradation to small fermentable
257 sugars, specifically glucose, especially in bottle 1, but lesser so in bottle 2 and

258 especially bottle 3.
259 Due to its stability, the beer in bottle 3 thus still gives a reasonable clue of
260 carbohydrate composition and content in lager beers from the beginning of the 20th
261 century. Maltotriose levels in bottle 3 (3.16 mg/ml) were more than 4-fold higher than
262 in a present-day reference (0.71 mg/ml), while maltotetraose was the most abundant
263 maltooligosaccharide in bottle 3 (3.9 mg/ml) and the present-day sample, albeit half
264 the concentration in the present-day sample (2.02 mg/ml) (Table 1). Not surprisingly
265 then, modern brewing methods have considerably improved the efficiency of raw
266 material usage. Notwithstanding, the HILIC traces of historic and present-day beers
267 show similar patterns of additional signals other than maltooligosaccharides. Thus,
268 variations in the composition of the most abundant sugars other than
269 maltooligosaccharides are limited, consistent with the similar profiles of limit dextrans
270 detected in ¹H NMR spectra.

271

272 **3.3 2D NMR-chemical detail of carbohydrate composition**

273 Highly resolved signatures of carbohydrate composition in the beer samples were
274 sought using homo- and heteronuclear 2D NMR spectroscopy. Figure 3A,B shows the
275 2D NMR detection of barley cell-wall carbohydrates, specifically arabinoxylan and β -
276 glucan in the historic beer samples. The beer of bottle 1 is largely devoid of
277 arabinoxylan and β -glucan, consistent with the action of microbial and/or enzymatic
278 activities in the bottle. Beers of bottles 2 and 3 contain arabinoxylan and β -glucan
279 signals that indicate a poor cleavage of these polysaccharides in beer production at the
280 turn of the last century, consistent with the fact that arabinoxylan and β -glucan pose
281 challenges for beer filterability throughout the 20th century and even today (Li, Lu,
282 Gu, Shi, & Mao, 2005).

283 Historic beer samples display various carbohydrate signals that are not found
284 in modern-style samples, or at considerably lower levels. Hence, the chemical
285 composition of carbohydrates and carbohydrate-adducts may be a possible aging
286 marker or may reflect the change in cereal varieties and malting practices since the
287 19th century (Ferrio, Alonso, Voltas, & Araus, 2006; D. W. Lachenmeier & Fügél,
288 2007; Linko, Haikara, Ritala, & Penttilä, 1998). Figures 3C and 4A indicate that
289 especially beer from bottle 1 contains glucans linked to primary alcohols that do not
290 derive from starch fragments (~4.93 ppm, at slightly lower frequency than the limit
291 dextrin $\alpha(1-6)$ branch points). Signal frequencies and sharp line widths indicate that
292 these signals arise from glucosides formed between α -anomeric glucose and low
293 molecular weight alcohols, for instance ethanol and glycerol. As the chemical
294 glycoside formation is acid catalyzed, while the beer from bottle 1 has the highest pH
295 of the historic beer samples, the formation of glucosides with low molecular weight
296 alcohols may result from enzymatic processes involving α -glucosidase instead. Such
297 an enzymatic, and hence specific mechanism, also seems plausible considering the
298 absence of corresponding β -glucosides in the spectral vicinity of gentiobiose (6-O- β -
299 D-glucopyranosyl-D-glucose, i.e. a glucopyranosyl β -linked to a primary alcohol
300 group at glucose C6, Figure 4B). Figure 4B indicates some further differences in
301 mono- and disaccharide composition of the beer samples and underlines the closest
302 resemblance of present-day lager beer by the beer of bottle 3, albeit with little
303 degradation of arabinoxylan to xylose and xylobiose fragments.

304

305 **3.4 Aging markers**

306 Beyond probing historical production conditions, the old beer samples were used to
307 validate and approximate limiting values of selected aging markers. The historic beer

308 samples were analysed using gas chromatographic separation coupled to mass
309 spectrometric analysis (GC-MS). Several volatile compounds, for instance acetate
310 esters (ethylacetate, isoamylacetate, phenylethyacetate), showed strongly reduced
311 abundance, down to less than 1% of present-day reference values. The decline of
312 these esters over more than a century could result from their high volatility and
313 presumably does not allow simple conclusions about the flavor profile of beer at the
314 onset of brewing science. In contrast, several aging markers (Vanderhaegen, Neven,
315 Verachtert, & Derdelinckx, 2006) were analysed, including Strecker aldehydes
316 (benzaldehyde, 3-methylbutanal) and Maillard aldehydes (furfural and 5-
317 methylfurfural), showing 3-fold to 40-fold higher values than in the present-day
318 reference (Table 2). Thus, these mentioned aldehydes in historic beer samples were
319 well suited to observe chemical reactivity during beer storage and were validated as
320 suitable aging markers. A comprehensive list of compounds analyzed by solid-phase
321 microextraction and GC-MS is provided in the supplemental Table S1.

322

323 **4. Discussion**

324

325 The use of pure culture lager yeast strains by Emil Christian Hansen revolutionized
326 lager beer production in the outgoing 19th century (Hansen, 1883). This first lager
327 beer yeast strain is known as *Saccharomyces carlsbergensis*, originally termed
328 *Unterhefe* No 1, and has been used in production since 1883 (Walther, Hesselbart, &
329 Wendland, 2014). Here, we analyse the molecular profiles of historic lager beer
330 samples intact beer bottles filled in the 1880s, 1890s and 1900s in comparison to
331 present-day references in order (1) to retrace the biotechnological challenges and
332 shortcomings in raw material handling at the onset of scientific brewing and (2) to

333 probe chemical, enzymatic and microbial reactivities during beer aging on historic
334 timescales.

335 The enzymatic remodeling of carbohydrates in complex mixtures is a hallmark
336 of many biotechnological production processes, including brewing. The carbohydrate
337 composition in the historic beer samples indicates an improved stability of the bottled
338 product from the 1880s to the 1900s. This finding is consistent with a growing
339 awareness of the importance of keeping wine and beer germ free following the work
340 of Pasteur in the 1860s and 1870s (Stewart & Russell, 1986). Two-dimensional NMR
341 spectroscopy shows that enzymatic activities in the supposedly oldest beer bottle have
342 led to the predominant degradation of maltooligosaccharides as well as barley
343 arabinoxylan and mixed linkage (1,3-1,4) β -glucan. Such degradation decreases in the
344 beer samples from the 1890s to the 1900s. The youngest historic beer sample from the
345 early 1900s contains most of its maltooligosaccharides in form of maltotriose and
346 maltotetraose and thus reflects the composition of present-day lager beers. The good
347 preservation of starch fragments in historic samples uniquely allows the comparison
348 with present-day samples and the identification of unconventional carbohydrates as
349 possible products of historic cereal varieties. For instance, maltotriose and
350 maltotetraose occur in the historic lager beer sample from the early 1900s at levels 2-
351 4-fold higher than for typical present-day lager beers (see literature averages in Table
352 1), thus reflecting the push towards optimized processes in present-day brewing. The
353 analysis of 10 recent lager beer samples of different years and sites from major
354 breweries (Table 1) revealed that all the present day samples had lower levels of
355 maltotriose and maltotetraose present in the historic lager beer sample from the early
356 1900s. This sample from the early 1900s has the lowest pH (4.2) and highest organic
357 acid content (Figure 1C) of the historic samples, while containing the least degraded

358 oligosaccharides and lowest monosaccharide concentrations. Hence, the
359 oligosaccharides can be considered chemically stable under weakly acidic conditions
360 in aqueous medium for more than a century.

361 Opposite to linear oligosaccharides, branched limit dextrans are found in all
362 historic beer samples at similar levels, and are evidently more resistant to amylase
363 degradation than maltooligosaccharides (Beeren, Petersen, Bojstrup, Hindsgaul, &
364 Meier, 2013). The limit dextrin structures in the historic samples are larger than those
365 found in present-day lager beer, again consistent with the improved usage of raw
366 materials in present-day lager beers, owing to the improved control over enzyme-
367 dependent processes during the course of the 20th century. When not degraded in
368 historic samples, the viscous cell wall polysaccharides arabinoxylan and mixed
369 linkage (1,3-1,4) β -glucan occur as large fragments rather than oligosaccharides, in
370 agreement with viscosity and filterability problems associated with these cell wall
371 polysaccharides throughout the 20th century and until today.

372 The detection of living, dormant *Saccharomyces carlsbergensis* yeast cells
373 and large amounts of alcoholic α -glycosides in the supposedly oldest sample is
374 consistent with the presence of enzymatic activities after bottling. In addition,
375 *Sporobolomyces roseus* (a beer spoilage yeast that is else not present in our lab) was
376 detected in the same bottle 1. Determinations of the yeast species were based on
377 rDNA sequencing. The presence of dormant yeast cells of 130 years of age in this
378 sample may be favored by the only weakly acid sample pH (pH 4.9). Yeast dormancy
379 in this sample occurs despite the presence of glucose above concentrations of 2 g/l,
380 presumably as the consequence of cytostasis due to nitrogen starvation, ethanol or
381 acidic pH. *Sporobolomyces* could have survived as spores, while *S. carlsbergensis* as
382 a triploid yeast has no tendency to sporulate. The use of polymerase chain reaction

383 validates the presence of *S. carlsbergensis* DNA in the sample (Walther, Hesselbart,
384 & Wendland, 2014).

385 Various carbohydrate signals are observed in historic beer samples that are
386 absent from the present-day reference. Due to their low concentration and the absence
387 of suitable reference compounds, these additional carbohydrate signals remain
388 hitherto unassigned. Markers of beer aging were determined in historic and present-
389 day samples in order to estimate the changes in beer chemical composition beyond
390 carbohydrates on historic timescales. Using gas chromatographic analysis, we find
391 that several volatile compounds are reduced to less than 1% of present-day reference
392 values. In contrast, several aging markers including Strecker aldehydes and
393 heterocycles showed 3-40-fold higher values than in the present-day reference.

394 In concluding, we analyse lager beer samples bottled shortly after the advent
395 of scientific brewing, including some of the first brews using pure lager yeast culture.
396 Historic beer samples contain most of the carbohydrates that are found in present-day
397 beer. Historic samples reflect an improving control over enzymatic polysaccharide
398 degradation processes at the end of the 19th century, yielding samples that are stable to
399 chemical, enzymatic and microbial degradation for more than a century. In contrast,
400 severe changes in chemical composition are found for esters and aldehydes. These
401 findings underline the stability of carbohydrate profiles and their utility in molecular
402 archaeology.

403

404 **Acknowledgements**

405 We thank Bjarne Maurer for providing access to the Carlsberg bottle collection and
406 insight into exact dating of bottles based on the respective labels used. This work was
407 funded by grant 2010_01_0135 of the Carlsberg Foundation to J.W.. S.M.

408 acknowledges Jens Duus and grant 2013_01_0709 of the Carlsberg Foundation for
409 funding.

410 **Conflict of interest statement**

411 The authors declare no conflicts of interest.

412

413 **References**

414

- 415 Beeren, S. R., Petersen, B. O., Bojstrup, M., Hindsgaul, O., & Meier, S. (2013). Time-
416 resolved in-situ observation of starch polysaccharide degradation
417 pathways. *Chembiochem*, *14*(18), 2506-2511.
- 418 Bøjstrup, M., Petersen, B. O., Beeren, S. R., Hindsgaul, O., & Meier, S. (2013). Fast
419 and accurate quantitation of glucans in complex mixtures by optimized
420 heteronuclear NMR spectroscopy. *Anal Chem*, *85*(18), 8802-8808.
- 421 Duarte, I. F., Barros, A., Almeida, C., Spraul, M., & Gil, A. M. (2004). Multivariate
422 Analysis of NMR and FTIR Data as a Potential Tool for the Quality Control
423 of Beer. *Journal of Agricultural and Food Chemistry*, *52*(5), 1031-1038.
- 424 Duarte, I. F., Godejohann, M., Braumann, U., Spraul, M., & Gil, A. M. (2003).
425 Application of NMR Spectroscopy and LC-NMR/MS to the Identification of
426 Carbohydrates in Beer. *Journal of Agricultural and Food Chemistry*, *51*(17),
427 4847-4852.
- 428 Ferreira, I. M. P. L. V. O. (2009). Beer Carbohydrates. *Beer in Health and Disease*
429 *Prevention, Preedy, V.R.*(Associated press Burlington, MA, USA).
- 430 Ferrio, J. P., Alonso, N., Voltas, J., & Araus, J. L. (2006). Grain weight changes over
431 time in ancient cereal crops: Potential roles of climate and genetic
432 improvement. *Journal of Cereal Science*, *44*(3), 323-332.
- 433 Hansen, E. C. (1883). Recherches sur la physiologie et la morphologie des
434 ferments alcooliques V. Methodes pour obtenir des cultures pures de
435 Saccharomyces et de mikroorganismes analogues. . *C. R. Trav. Lab.*
436 *Carlsberg*, *2*, 92-105.
- 437 Hughes, S., & Johnson, D. C. (1982). High-performance liquid chromatographic
438 separation with triple-pulse amperometric detection of carbohydrates in
439 beverages. *Journal of Agricultural and Food Chemistry*, *30*(4), 712-714.
- 440 Lachenmeier, D., Frank, W., Humpfer, E., Schäfer, H., Keller, S., Mörtter, M., &
441 Spraul, M. (2005). Quality control of beer using high-resolution nuclear
442 magnetic resonance spectroscopy and multivariate analysis. *European*
443 *Food Research and Technology*, *220*(2), 215-221.
- 444 Lachenmeier, D. W., & Fügel, D. (2007). Reduction of Nitrosamines in Beer -
445 Review of a Success Story. *BrewingScience*, *60*, 84-89.
- 446 Laine, R. A. (1994). Invited Commentary: A calculation of all possible
447 oligosaccharide isomers both branched and linear yields 1.05×10^{12}
448 structures for a reducing hexasaccharide: the Isomer Barrier to
449 development of single-method saccharide sequencing or synthesis
450 systems. *Glycobiology*, *4*(6), 759-767.
- 451 Li, Y., Lu, J., Gu, G., Shi, Z., & Mao, Z. (2005). Studies on water-extractable
452 arabinoxylans during malting and brewing. *Food Chem*, *93*(1), 33-38.
- 453 Libkind, D., Hittinger, C. T., Valerio, E., Goncalves, C., Dover, J., Johnston, M.,
454 Goncalves, P., & Sampaio, J. P. (2011). Microbe domestication and the
455 identification of the wild genetic stock of lager-brewing yeast. *Proc Natl*
456 *Acad Sci U S A*, *108*(35), 14539-14544.
- 457 Linko, M., Haikara, A., Ritala, A., & Penttilä, M. (1998). Recent advances in the
458 malting and brewing industry1. *Journal of Biotechnology*, *65*(2-3), 85-98.

459 Marcone, M. F., Wang, S., Albabish, W., Nie, S., Somnarain, D., & Hill, A. (2013).
460 Diverse food-based applications of nuclear magnetic resonance (NMR)
461 technology. *Food Research International*, 51(2), 729-747.

462 McGovern, P. E., Zhang, J., Tang, J., Zhang, Z., Hall, G. R., Moreau, R. A., Nunez, A.,
463 Butrym, E. D., Richards, M. P., Wang, C. S., Cheng, G., Zhao, Z., & Wang, C.
464 (2004). Fermented beverages of pre- and proto-historic China. *Proc Natl*
465 *Acad Sci U S A*, 101(51), 17593-17598.

466 Meussdoerffer, F. G. (2009). A Comprehensive History of Beer Brewing. In
467 *Handbook of Brewing*, (pp. 1-42): Wiley-VCH Verlag GmbH & Co. KGaA.

468 Nord, L. I., Vaag, P., & Duus, J. O. (2004). Quantification of organic and amino
469 acids in beer by ¹H NMR spectroscopy. *Anal Chem*, 76(16), 4790-4798.

470 Petersen, B. O., Hindsgaul, O., & Meier, S. (2014). Profiling of carbohydrate
471 mixtures at unprecedented resolution using high-precision ¹H-¹³C
472 chemical shift measurements and a reference library. *Analyst*, 139(2),
473 401-406.

474 Petersen, B. O., Motawie, M. S., Moller, B. L., Hindsgaul, O., & Meier, S. (2014).
475 NMR characterization of chemically synthesized branched alpha-dextrin
476 model compounds. *Carbohydr Res*.

477 Petersen, B. O., Nilsson, M., Bojstrup, M., Hindsgaul, O., & Meier, S. (2014). (¹H
478 NMR spectroscopy for profiling complex carbohydrate mixtures in non-
479 fractionated beer. *Food Chem*, 150, 65-72.

480 Rodrigues, J. A., Barros, A. S., Carvalho, B., Brandão, T., & Gil, A. M. (2011).
481 Probing beer aging chemistry by nuclear magnetic resonance and
482 multivariate analysis. *Analytica Chimica Acta*, 702(2), 178-187.

483 Saison, D., De Schutter, D. P., Delvaux, F., & Delvaux, F. R. (2008). Optimisation of
484 a complete method for the analysis of volatiles involved in the flavour
485 stability of beer by solid-phase microextraction in combination with gas
486 chromatography and mass spectrometry. *Journal of Chromatography A*,
487 1190(1-2), 342-349.

488 Sicard, D., & Legras, J.-L. (2011). Bread, beer and wine: Yeast domestication in
489 the *Saccharomyces sensu stricto* complex. *Comptes Rendus Biologies*,
490 334(3), 229-236.

491 Stewart, G. G., & Russell, I. (1986). One hundred years of yeast research and
492 development in the brewing industry. *Journal of the Institute of Brewing*,
493 92(6), 537-558.

494 Vanderhaegen, B., Neven, H., Verachtert, H., & Derdelinckx, G. (2006). The
495 chemistry of beer aging – a critical review. *Food Chem*, 95(3), 357-381.

496 Walther, A., Hesselbart, A., & Wendland, J. (2014). Genome sequence of
497 *Saccharomyces carlsbergensis*, the world's first pure culture lager yeast.
498 *G3 (Bethesda)*, 4(5), 783-793.

499 Wilhelmson, A., Londesborouhg, J., & Juvonen, R. (2012). Analysing the
500 shipwreck beer.
501 [http://www.vtt.fi/files/news/2012/Hylkyolut_Maarianhamina_1005201](http://www.vtt.fi/files/news/2012/Hylkyolut_Maarianhamina_1005201_2_Wilhelmson.pdf)
502 [2_Wilhelmson.pdf](http://www.vtt.fi/files/news/2012/Hylkyolut_Maarianhamina_1005201_2_Wilhelmson.pdf).
503

504 **Figure Captions**

505

506 **Figure 1.** (A) Historic bottles from the Carlsberg Museum bottle collection filled with
507 original lager beer. Time ranges for their filling were deduced from the names of
508 fillers given on the labels. (B) ^1H NMR spectrum of the spectral region containing α -
509 anomeric sugar signals from starch fragments in the present-day reference and historic
510 beer samples of the bottles shown in (A). The hump at 5.35 ppm results from $\alpha(1-4)$
511 signals near branch points. Horizontal lines designate $\alpha(1 \rightarrow 4)$ glycosidic bonds, vertical
512 lines designate $\alpha(1 \rightarrow 6)$ glycosidic bonds and circles designate glucopyranosyl units, where
513 the filled circle yields the signal in a structural motif indicated by open circles. (C) ^1H NMR
514 spectrum of the aliphatic spectral region. Increased organic acid content, especially of
515 lactate and acetate, is evident, while amino acids content does not appear significantly
516 increased. Residual d_2 -acetate signal derives from addition of d_3 -acetate for stabilizing
517 sample pH and internal referencing. (D) ^1H NMR spectrum of the aromatic spectral
518 region, showing increasing formic acid content in historic samples 1-3 and the
519 absence of broad protein background signals in the historic samples due to protein
520 hydrolysis or precipitation. The ^1H NMR spectra are normalized relative to an internal
521 residual d_2 -acetate standard signal.

522

523 **Figure 2.** HILIC traces of 2-aminobenzamide labeled beer samples for the
524 quantitation of glucose, maltose and maltooligosaccharides in historic samples and a
525 present-day reference. Absolute quantitations were derived using a mixture of
526 standard compounds. Quantitations are tabulated in Table 1. G1-G6 represent $\alpha(1-4)$
527 linked glucans with dp 1 (glucose) to dp 6 (maltohexaose).

528

529 **Figure 3.** (A) ^1H - ^1H - COSY and (B) ^1H - ^{13}C HSQC spectra of barley cell wall
530 polysaccharides in the historic beer samples. The spectra indicate nearly complete
531 degradation in bottle 1, presumably after bottling, and intact β -glucopyranosyl (red)
532 and β -xylopyranosyl (orange) polysaccharide chains with little chain cleavage in
533 beers from bottles 2 and 3. In depictions of arabinoxylan fragments, squares designate
534 arabinofuranosyl units, circles designate xylopyranosyl-units, horizontal lines indicate
535 β -(1 \rightarrow 4) glycosidic bonds, vertical lines indicate α -(1 \rightarrow 2) glycosidic bonds, and
536 diagonal lines indicate α -(1 \rightarrow 3) glycosidic bonds. In depiction of β -glucan structures,
537 circles designate glucopyranosyl units, horizontal lines indicate β -(1 \rightarrow 4) glycosidic
538 and diagonal lines indicate β -(1 \rightarrow 3) glycosidic bonds. (C) Presence of alcoholic α -
539 glycosides in beer from bottle 1 at ^1H - ^{13}C HSQC spectral positions indicated by
540 arrows. Ethyl- α -glucopyranoside was identified using an enzymatically synthesized
541 reference compound.

542

543 **Figure 4.** (A) ^1H - ^{13}C HSQC spectra of historic and present-day lager beer samples.
544 Spectral regions corresponding to starch fragments are highlighted in bottle 1 and
545 regions corresponding to β -glucan and β -xylan for sample bottle 2. Additional signals
546 that are not present in present-day lager samples, or at lesser amounts, are highlighted
547 by a grey area in sample bottle 3. (B) Spectral region highlighting differences in
548 mono- and disaccharide compositions of historic and present-day lager beer samples.

549

550

551 **Tables**552 **Table 1.** Glucose and maltooligosaccharide content of historic beer samples in g/l.

Sample	Glucose	Maltose	Maltotriose	Malto- tetraose	Malto- pentaose	Malto- hexaose
Bottle 1	2.54	0.13	0.04	0.05	nd	nd
Bottle 2	1.86	1.48	2.03	0.33	nd	0.04
Bottle 3	0.36	0.70	3.16	3.90	0.80	0.41
ref	0.02	0.39	0.71	2.02	0.33	nd
ref av^a	0.07±0.03	0.15±0.26	0.46±0.36	1.51±0.60	0.39±0.20	0.19±0.08
lit av^b	nd	0.05±0.10	1.25±0.71	3.45±1.31	nd	nd

553 ^aaverage determination by high-performance anion exchange chromatography with
554 pulsed amperometric detection (as described in (Bøjstrup, Petersen, Beeren,
555 Hindsgaul, & Meier, 2013)) of 10 contemporary commercial lager beer samples of
556 major brewing companies. The analysis includes samples of the years 2011-2014,
557 including sample variation from different sites.

558 ^baverage determination by HPLC analyses of 18 lager beers reported in the literature
559 (Ferreira, 2009).

560

561 **Table 2.** Strecker aldehydes and heterocyclic aldehydes produced during beer aging,
562 reported as parts per billion (w/v) as determined by GC-MS analysis.

Sample	Furfural	5-Methyl furfural	Benzaldehyde	3-Methyl butanal
Bottle 1	70.38	7.76	30.17	7.72
Bottle 2	143.91	10.74	138.91	9.83
Bottle 3	401.69	18.91	26.89	5.14
ref	14.44	0.56	10.87	nd

563