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Application of NMR Spectroscopy and LC-NMR/MS to the Identification of Carbohydrates in Beer

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The application of LC-NMR/MS for the direct identification of carbohydrates in beer has been studied. Carbohydrates are major beer components, and their structural characterization by NMR alone is seriously hindered by strong spectroscopic overlap. Direct analysis of beer by LC-NMR/MS enables the rapid (1−2 h) identification of dextrins with degree of polymerization (DP) of up to nine monomers, with degassing being the only sample treatment required. Although the presence of α(1→6) branching points is easily indicated by NMR for each subfraction separated by LC, difficulties arise for the unambiguous assignment of linear or branched forms of high DP dextrins. The two beer samples investigated in this work were found to have significantly different oligosaccharide compositions, reflecting the different production conditions employed. The use of hyphenated NMR for the rapid characterization of the carbohydrate composition of beers may be the basis of a useful tool for the quality control of beer.

KEYWORDS: Beer; NMR; LC-NMR/MS; carbohydrate; dextrins; composition

INTRODUCTION

Beer is a very complex mixture, containing a vast number of metabolites widely ranging in nature and in concentration level. In addition to the natural composition of this fermented beverage made from malted grains (usually barley), hops, yeast, and water, a great variety of other ingredients such as fruits, herbs, and spices may also be added to give beer a specific aroma/flavor. The different combinations of ingredients, production processes, and storage conditions result in an enormous variety of beers, differing strongly in their chemical composition, and hence, in their organoleptic characteristics (*1*). It is clear that an adequate characterization of beer composition could be potentially correlated to origin and quality. Therefore, the development of rapid and accurate analytical methods, with the ability to account for a range of compounds as comprehensive as possible, would be of significant importance for the quality control of beer and other foodstuffs, therefore being of value for both producers and consumers. In addition, beer is a potentially important source of natural products, such as antioxidants, flavors, aroma-inducers, and emulsion-stabilizers, which are of great interest to a wide scientific community that aims at evaluating the potential of new compounds in various areas such as health, nutrition, and food quality/functionality.

In addition to water and ethanol, major beer components are carbohydrates comprising fermentable sugars (e.g., glucose, maltose, and maltotriose) as well as glucose oligosaccharides (dextrins) and arabinoxylans. Fermentable sugars directly contribute to the sweetness of beer, whereas carbohydrates with more than four glycosyl units can be beneficial to the perception of beer in that they contribute to body or mouthfeel (*1*). Monitoring of beer carbohydrate composition may therefore be an important tool for modern brewing technology, particularly in the selection of raw materials and yeast strains, product development (e.g., low caloric beers) and quality control. Carbohydrates have been determined in some beers by chemical and enzymatic analysis (*2*) and by chromatography (*3*−*5*), their nature and proportion being found to depend significantly on beer type (*2*, *5*). Other components are also very important for the quality of beer, for instance proteins and amino acids influence beer foam and haze stability, whereas phenolics such as cinnamic and benzoic acids, catechins, and flavonols contribute to beer flavor, physical stability, and antioxidant activity. Much work has been devoted to characterizing different families of beer components, mainly using methods such as chemical and enzymatic analysis (*2*), chromatography (*3*−*7*), electrophoresis (*8*), LC-MS (*9*, *10*), and NMR spectroscopy of beer fractions (*11*, *12*). Usually, these analytical techniques are based on some kind of sample pretreatment (e.g., separation and concentration) and an adequate choice of protocol, depending on the specific family/type of compounds under analysis. Recent work (*13*) has shown that NMR spectroscopy enables a direct and rapid overview of beer chemical composition to be obtained, without the need of pretreatment other than degassing. The assignment of 1- and 2-dimensional NMR spectra of beer

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samples enabled the identification of about 30 compounds ranging from organic acids, amino acids, and alcohols to higher molecular weight compounds such as lipids and large aromatic compounds, possibly polyphenolics. However, the identification of many compounds in all regions, and in particular of carbohydrates (detected in the 3.0−6.0 ppm region), is hindered by strong signal overlap. An additional difficulty arises from the requirement for presaturation of ethanol resonances at 1.17 and 3.64 ppm, as well as that of water at 4.77 ppm, which results in many overlapped and neighboring resonances becoming suppressed or significantly diminished.

In this work, the application of LC-NMR/MS to the direct analysis of beer has been explored for the first time to our knowledge, aiming at showing the potential of the method to characterize beer carbohydrate composition. The hyphenated technique of LC-NMR has already been used for the identification of hop bitter acids (*12*), having demonstrated the utility of coupling a first LC step with the NMR characterization of selected chromatographic fractions. In the present paper, MS spectrometry is used in parallel to LC-NMR, so that the three sets of experimental data (retention times, ^{1}H NMR spectra, and MS spectra) may be recorded in a period that may be as short as 1 or 2 h, providing large amounts of structural information. Beer samples of different types (lagers or ales) and labels were compared in relation to their carbohydrate profile in the 1D NMR spectra, and two samples representative of significantly different profiles were selected for LC-NMR/MS analysis.

MATERIALS AND METHODS

Samples. Some of the beer samples studied in this work were obtained commercially, while others were kindly provided by Brewing Research International, UK. The samples considered here comprise one ale beer (top fermented) and two lager beers (bottom fermented) of different labels; these beer samples are named throughout this work as ale 1, lager 1, and lager 2, respectively. Out of these samples, ale 1 and lager 2 were selected for the LC-NMR/MS characterization due to their distinct NMR profiles in the carbohydrate region. For NMR analysis, the beer samples were degassed for 10 min in an ultrasonic bath and prepared to contain 10% D_2O and 0.02% sodium 3-(trimethylsilyl)-propionate-d_4 (TSP-d_4) as chemical shift reference. For LC-NMR/MS carbohydrate characterization, the beers were simply degassed before injection.

NMR and LC-NMR/MS Measurements. NMR spectra (1D and 2D) of the beer samples were recorded on a Bruker Avance DRX-500 spectrometer, operating at 500.13 MHz for proton. The ^{1}H 1D spectra were acquired using a pulse sequence based on the 2-dimensional NOE experiment (*14*), with a 90° pulse of 8.5 μs. Water (4.77 ppm) and ethanol signals (1.17 and 3.64 ppm) were suppressed by applying a modulated shaped pulse, with triple offset and amplitude scaling, during relaxation delay and mixing time (100 ms). Transients ($n = 128$) were collected into 16K data points, with a spectral width of 5482 Hz. The TOCSY spectra were acquired in the phase sensitive mode, using time proportional phase incrementation (TPPI); the MLEV17 pulse sequence and a modulated shaped pulse for presaturation of water and ethanol resonances were applied (*15, 16*). Sixteen scans were collected for each of the 512 increments in the f_1 dimension, using a spectral width of 5482 Hz in both dimensions, 2K data points, a mixing time of 100 ms, and a relaxation delay of 1.5 s.

For the LC-NMR/MS studies, the HPLC system consisted of an HP 1100 solvent delivery pump with vacuum degasser (Agilent, Waldbronn, Germany), a manual injector from Rheodyne, Model 7725i (Cotati, CA), equipped with a 1-mL sample loop, and a diode array detector from Bruker BioSpin (Rheinstetten, Germany). A BPSU-36 interface coupled to a DRX-500 NMR spectrometer equipped with a ^{1}H-^{13}C inverse-detection flow probe (cell of 4 mm i.d. with an active volume of 120 μL) from Bruker BioSpin was used. For on-line MS detection, an ESQUIRE−3000 ion trap mass spectrometer, equipped with an electrospray ion source, from Bruker Daltonics (Bremen, Germany) was used. Five percent of the eluent was split into the MS using a splitter from LC Packings (San Francisco, CA), which was part of an LC-NMR/MS interface from Bruker BioSpin. Both UV as well as MS were used to trigger the transfer of the HPLC fractions to a BPSU-36 peak sampling unit (Bruker BioSpin) for temporary storage or direct transfer to the NMR.

For identification of beer carbohydrates, chromatographic separation was carried out at 35 °C, using a 300 × 7.8-mm cation-exchange ION-300 column (Interaction Chromatography Inc., CA), with a particle size of 5.0 μm, at a flow rate of 0.3 mL/min, and a diode array detection at 200 and 220 nm. The injection volume was 100 μL, and the mobile phase consisted of 0.0085N H_2SO_4 in D_2O. ^{1}H NMR spectra were recorded at 500 MHz, using the on-flow and the loop-sampling modes. For on-flow experiments, successive NMR spectra were acquired automatically, co-adding 16 transients and using 8K data points with a spectral width of 5000 Hz. Acquisition time was 0.82 s, with 1.8 s delay between transients. A pulse sequence with presaturation during relaxation delay was used (sequence 'lc2pr' from the Bruker pulse program library). 1D ^{1}H NMR spectra of loops were recorded using multiple solvent suppression with time-shared double presaturation of water and acetonitrile ^{1}H frequencies by means of a shaped low-power RF-pulse and CW decoupling on the F2 channel for the decoupling of the ^{13}C satellites of residual acetonitrile (used to wash the system, and thus, detected even when using 100% D_2O for the elution). 128 transients were collected into 32K computer data points, with a spectral width of 10000 Hz and an acquisition time of 1.6 s. Prior to Fourier transformation, an exponential multiplication was applied to the FID, corresponding to a line broadening of 1 Hz. In addition to the 1D spectra, phase-sensitive TOCSY experiments with WET (water suppression enhanced through T_1 effects) solvent suppression (*17*) and ^{13}C decoupling during WET-gradient sequence and acquisition were carried out. A total of 16 increments with 128 transients and 2K computer data points were acquired in simultaneous mode with a spectral width in both dimensions of 10 000 Hz. The applied mixing time of the MLEV spin lock was 65 ms. The data were apodized with a shifted squared sine bell window function 2.5 Hz in both dimensions and zero-filled in the f_1 dimension to 1024 data points. Electrospray ionization (ESI) was carried out in positive-ionization mode, and mass spectra were acquired up to m/z 3000, after adding 50 μL/min of 20 mM aqueous sodium acetate solution to the split ratio of the eluent via a T-piece with a syringe pump.

RESULTS AND DISCUSSION

Figure 1 shows the ^{1}H NMR spectrum of a beer sample recorded at 500 MHz. The assignment of many of the peaks observed, including some of the aromatic peaks that have relatively lower intensities (**Figure 1**, enlargement), has been reported in a recent publication (*13*), which also acknowledged the problem of carbohydrate identification due to signal overlap for most glucose oligomers. Such oligomers arise from the breakdown of starch polymers, thus giving rise to linear dextrins formed from amylose or amylopectin, as well as branched dextrins formed from amylopectin. The sugar regions of the ^{1}H NMR spectra of one ale beer and two lager beers are shown in **Figure 2**. The spectra of ale 1 and lager 1 are remarkably similar both in terms of resolution and spectral profile, showing that ale and lager beer types do not necessarily differ significantly in their carbohydrate composition, in agreement with previously reported work (*13*). The relatively low resolution seen for these samples (**Figure 2**, parts A and B) reflects the predominance of oligomeric and/or polymeric carbohydrates undergoing slow molecular tumbling and hence leading to faster transverse relaxation and broader spectra. This broadening effect quickly reveals itself as the oligomer size increases, as illustrated by the spectra of standard carbohydrates shown in **Figure 3**. Furthermore, the assignment shown in the spectra of standards

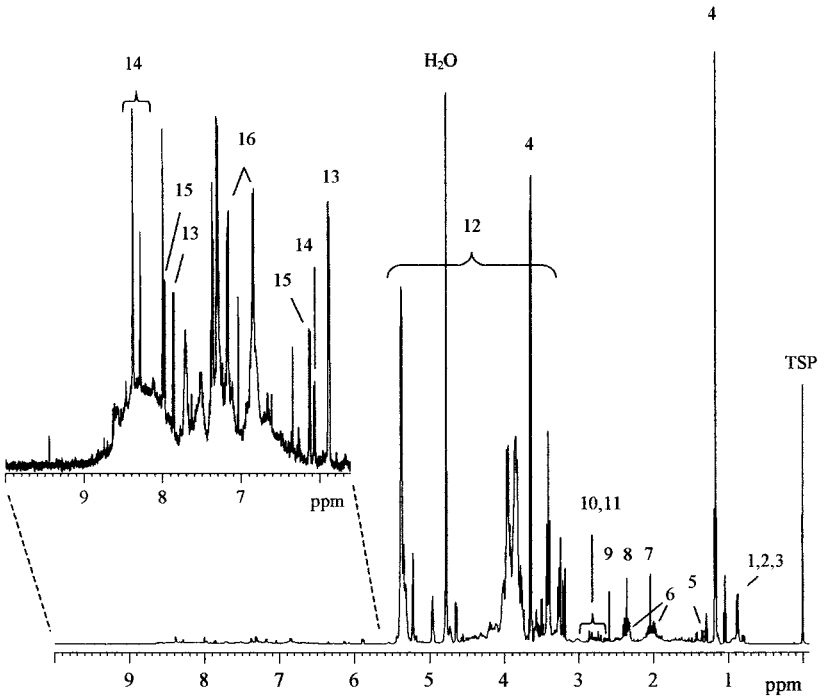

Figure 1. [1]H NMR spectrum of a beer sample recorded at a 500-MHz field strength. Some assignments are indicated: 1-propanol, 2-isobutanol, 3-isopentanol, 4-ethanol, 5-lactic acid, 6-proline, 7-acetic acid, 8-pyruvic acid, 9-succinic acid, 10-citric acid, 11-malic acid, 12-dextrins, 13-uridine, 14-adenosine/inosine, 15-cytosine, and 16-tyrosine.

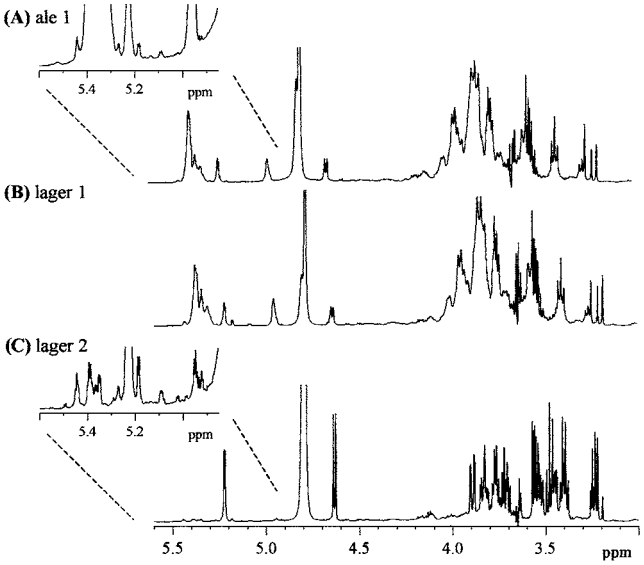

Figure 2. Sugar regions (3.0–5.5 ppm) of the [1]H NMR spectra of (A) one ale beer, ale 1, and two lager beers, (B) lager 1, and (C) lager 2. The inserts show the additional peaks observed in the anomeric regions.

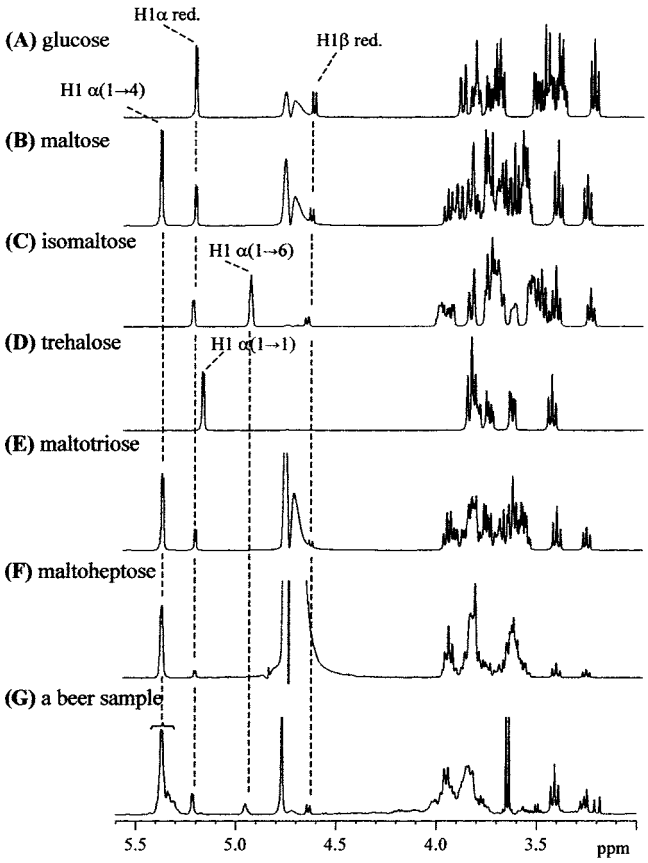

Figure 3. [1]H NMR spectra (500 MHz) of standard carbohydrates differing in size and structure: (A) glucose, (B) maltose, (C) isomaltose, (D) trehalose, (E) maltotriose, (F) maltoheptose, and (G) a beer sample. The assignment of signals in the anomeric region (4.5–5.5 ppm) is shown, with H1α red. and H1β red. indicating the reducing anomeric protons.

helps in interpreting beer spectra in terms of the extent of branching of the composing carbohydrates. Indeed, the beer profile in **Figure 3G** clearly shows the presence of α(1→6) branched carbohydrates, and the broad overlap of peaks at 5.3–5.4 ppm expresses a large spread in nonreducing α(1→4) sites due to the different-sized linear glucose segments present. The spectrum of lager 2 (**Figure 2C**), on the other hand, shows a well-resolved sugar region, reflecting the predominance of low molecular weight carbohydrates, namely glucose. Comparison of this sample with lager 1 (**Figure 2B**) thus indicates that the lager type may comprise beers with very distinct carbohydrate profiles. In addition, for all beer samples, the vertical expansions of the anomeric spectral regions (**Figure 2**) show that additional, less abundant compounds, presumably oligosaccharides, are present in addition to the predominant compounds. However, the strong overlap in the sugar region, observed even in the 2D

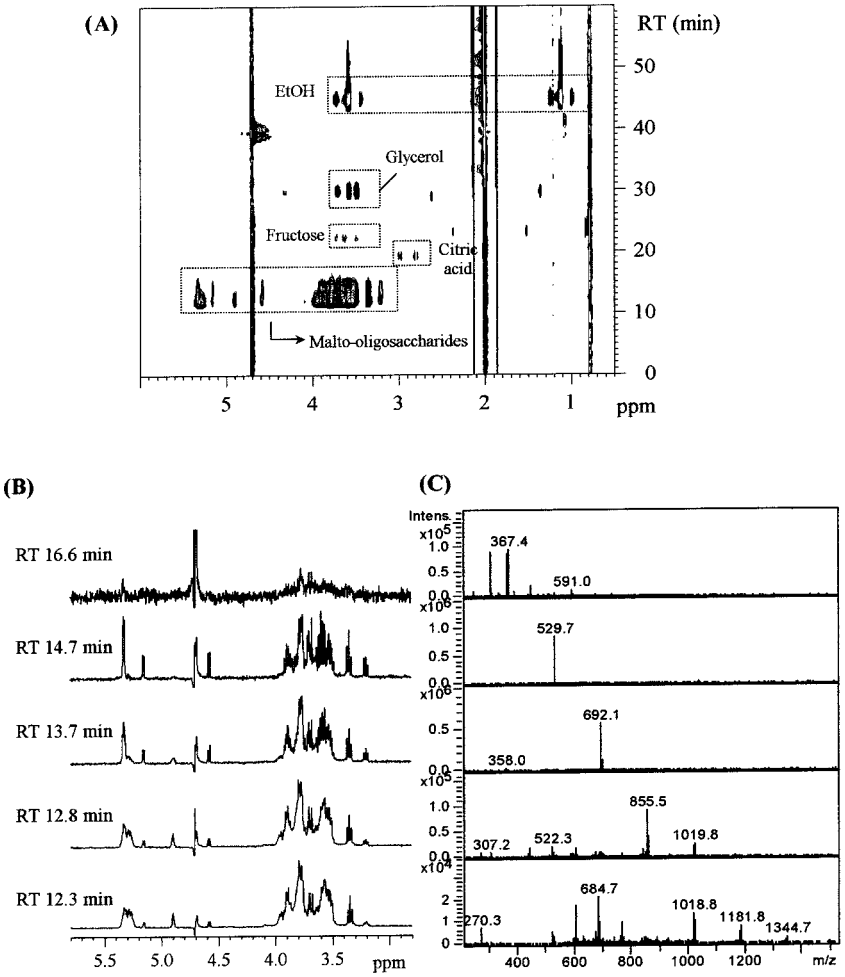

Figure 4. (A) On-flow record obtained for ale 1. The labels identify the main separated fractions, (B) rows extracted from the on-flow record, (C) MS spectra acquired concurrently with the NMR data, using positive-ionization.

spectra of beer (not shown), hinders further carbohydrate assignment, a problem which calls either for the traditional approach of sample simplification by extraction and concentration or for the development of alternative methods for the analysis of the whole foodstuff.

In this work, hyphenated NMR was applied to the direct analysis of two beer samples, showing clearly different sugar profiles in the ^{1}H NMR spectra, ale 1, and lager 2 (**Figure 2**, parts A and C), aiming at the characterization of their carbohydrate composition. The results obtained for ale 1, richer in glucose oligosaccharides, are presented first, followed by their comparison with those obtained for lager 2, in which monomeric glucose predominates. The ^{1}H NMR-detected HPLC on-flow record obtained for ale 1 is shown in **Figure 4**A and the compounds identified are listed in **Table 1**. Some components were readily separated and identified through their NMR spectra, namely ethanol (RT 45.0 min), glycerol (RT 30.0 min), fructose (RT 22.5 min), and citric acid (RT 19.5 min). It should be noted that the detection of glycerol by NMR (at 3.48, 3.57, and 3.70 ppm) is usually hindered by overlap with sugar signals and by the effects of ethanol suppression at 3.64 ppm. The major carbohydrates of ale 1 elute at retention times between 12 and 17 min and show poor separation, as expected based on their very similar sizes and structures. **Figure 4B** shows some rows extracted from the on-flow record in that range of retention times. The changes in spectroscopic resolution and profiles are consistent with the elution of malto-oligosaccharides of different sizes and structures, with the larger branched components eluting first, followed by smaller compounds. Integration of selected anomeric signals may give estimates of the average number of glucose units and branching points per molecule (*18*), as long as the T_1 relaxation times of the molecules are shorter than the recycle time employed. The average number of glucose rings per molecule is given by the ratio of the summed areas of H1 protons in both α(1→4) and α(1→6) glycosidic linkages (signals at 5.30−5.40 and 4.9−5.0 ppm, respectively) and the summed areas for the reducing H1 protons (5.22 and 4.63 ppm for the α and β anomers, respectively). The average number of branching points per molecule is estimated by the ratio of the integral of the peak for H1 protons in α(1→6) linkages (4.9−5.0 ppm) and that of the peak for H1 protons in α(1→4) linkages (5.3−5.4 ppm), multiplied by the number of total linkages. The results of these calculations are shown in **Table 1** and are discussed with the MS data corresponding to each of the on-flow lines selected for study (**Figure 4C**).

The MS results presented in **Table 1** were obtained in the positive-ionization mode, using a sodium acetate-containing mobile phase to facilitate ionization. The MS analysis of the fraction eluting at 12.3 min gave rise to both mono- and doubly charged cations, $[M+Na]^+$ and $[M+2Na]^{2+}$, for oligosaccharides with *m*/*z* values corresponding to DP (degree of polymerization) of 6, 7, and 8. For instance, the peak at *m*/*z* 1345 results from ionization with sodium (atomic weight 23) of DP8 (M_w 1315) in the partially deuterated form (in this case, involving seven deuterium sites). It should also be noted that the multiplet nature of the MS peaks arises from deuteration of

Table 1. NMR and MS Data Obtained for the Carbohydrates Identified in Ale 1 by LC-NMR/MS

	NMR		MS (positive-ionization)		
RT (min)	avg no. glucose units/molecule[a]	avg no branchings/molecule	*m/z*	molecular ion[b]	cpds identified (M_w)
12.3	9.6	1.4	522	$[DP6 + 2Na]^{2+}$	
			604	$[DP7 + 2Na]^{2+}$	DP6 (M_w 990.9),
			685	$[DP8 + 2Na]^{2+}$	DP7 (M_w 1153.1),
			766	$[DP9 + 2Na]^{2+}$	DP8 (M_w 1315.2),
			1019	$[DP6 + Na]^{+}$	and DP9 (M_w 1477.4)
			1182	$[DP7 + Na]^{+}$	
			1345	$[DP8 + Na]^{+}$	
12.8	7.0	0.9	439	$[DP5 + 2Na]^{2+}$	DP5 (M_w 828.8)
			522	$[DP6 + 2Na]^{2+}$	and DP6 (M_w 990.9)
			856	$[DP5 + Na]^{+}$	
			1020	$[DP6 + Na]^{+}$	
13.7	4.2	0	358	$[DP4 + 2Na]^{2+}$	maltotetraose
			692	$[DP4 + Na]^{+}$	(M_w 666.6)
14.7	3.2	0	530	$[DP3 + Na]^{+}$	maltotriose
					(M_w 504.5)
16.6	S/N improved in loop spectrum		367	$[DP2 + Na]^{+}$	maltose and trehalose
					(M_w 342.3)
22.5	fructose profile		204	$[hexose + Na]^{+}$	fructose
					(M_w 180.2)

[a] Calculated by integration of anomeric signals as described in text, S/N = signal-to-noise ratio. [b] DP = degree of polymerization.

the carbohydrate hydroxyl groups to different extents. DP9 was identified in the same fraction through the formation of only its disodium form. This is in agreement with previous mass spectrometry studies of oligosaccharides (*19*), which indicated the formation of disodium cations for oligomers with DP > 4. The MS results alone do not enable the unambiguous identification of the oligomers as branched or linear; in this respect, the corresponding NMR row (**Figure 4B**) does indicate, through the 4.96 ppm peak, that at least some of the dextrins identified comprise α(1→6) branches. However, more complete identification might be achieved through a combination of specific chromatographic methods optimized for oligosaccharide separation and multidimensional NMR analysis (*3*, *11*, *18*) of the 12.3 min subfraction. The 12.8 min subfraction shows the presence of DP6 and DP5, viewed by the identification of both mono and disodium cations for each oligosaccharide. The corresponding NMR spectrum indicates branching of these carbohydrates to some extent. The following subfraction under study, eluted at 13.7 min, was found to comprise mainly DP4 oligosaccharides which from the NMR spectrum are shown to be in the linear form, that is, maltotetraose. Indeed, the residual broad signal at 4.96 ppm seems to result from the gradual decrease of the broad components in the spectrum (note the low intensity at 5.25−5.30 ppm), rather than from the predominant DP4 oligomer present. At 14.7 min, maltotriose is clearly identified, and finally, at 16.6 min, a fraction with very low signal-to-noise ratio in the NMR dimension gives an MS peak characteristic of a glucose disaccharide. The unambiguous identification of this disaccharide was only possible by recording a higher number of transients for the collected 16.6 min subfraction (**Figure 5A**) and comparing the resulting spectrum to those of standard solutions (**Figure 3**). This enabled the identification of maltose and trehalose.

The on-flow record obtained for lager 2 reveals a significantly different carbohydrate composition clearly dominated by glucose, as well as the presence of glycerol, fructose, and citric acid (**Figure 6A**). The oligomers that give rise to weak peaks in the sugar region of the 1D spectrum (**Figure 2C**) are found to elute at earlier retention times (<20 min for a flow rate of 0.3 mL/min), with a satisfactory degree of separation but with very poor signal-to-noise ratio. In this case, the oligomers

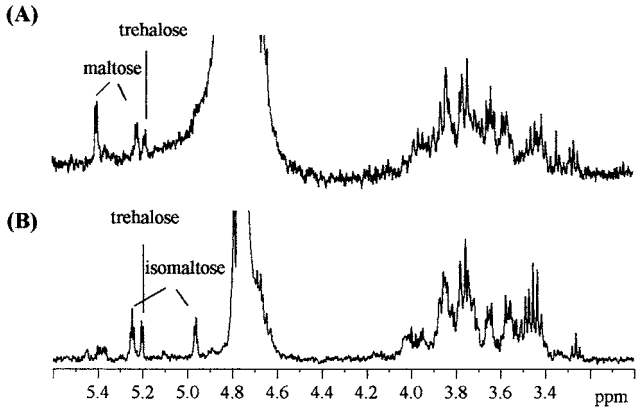

Figure 5. (A) Spectrum of the subfraction collected at retention time 16.6 min for ale 1 and (B) spectrum of the subfraction collected at retention time 17.3 min for lager 2.

identified by MS ranged only from DP2 to DP4 (**Table 2**). For the DP4 fraction eluting at 13.5 min, no confirmation of the existence of a branching point could be achieved due to the low signal-to-noise ratio in the NMR (**Figure 6B**). However, the disaccharide-containing subfraction, eluting with highest signal-to-noise ratio at 17.3 min, was collected to allow the coaddition of transients (**Figure 5B**). By comparison with standard solutions, this procedure enabled the identification of isomaltose and trehalose as the disaccharides present.

In conclusion, this work has shown that LC-NMR/MS enables the rapid (1−2 h) successful identification of dextrins with up to nine monomers by the direct analysis of intact beer samples, with degassing being the only sample treatment required. Although the presence or absence of α(1→6) branching points in each subfraction may be indicated by NMR, unambiguous assignment of higher DP dextrins in terms of their form, linear or branched, is not easily achieved without further improvement of the chromatographic methods/conditions used, in tandem with NMR multidimensional methods. The two beer labels investigated in this work were found to have a rather different oligosaccharide composition, which should reflect the different ingredients and/or production conditions employed. Future studies will include further analysis of different beer types, as

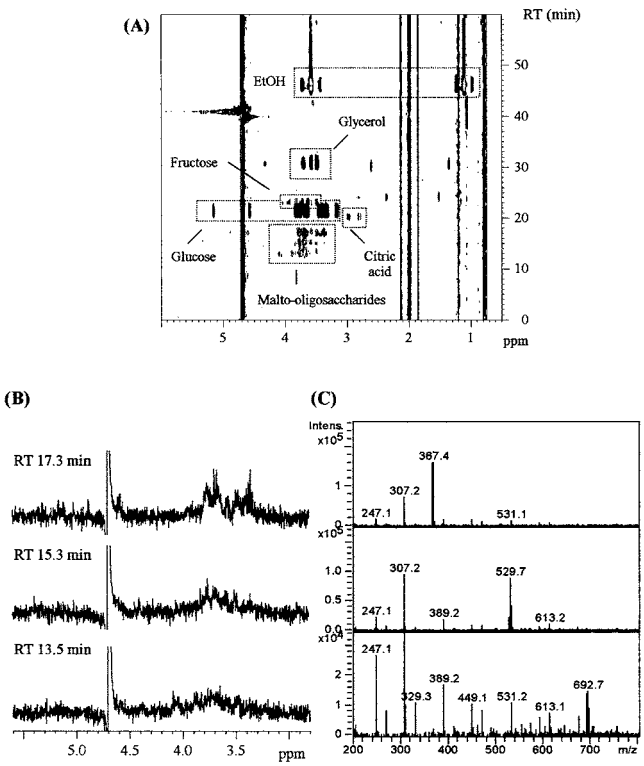

Figure 6. (A) On-flow record obtained for lager 2. The labels identify the main separated fractions, (B) rows extracted from the on-flow record, (C) MS spectra acquired concurrently with the NMR data, using positive-ionization.

Table 2. NMR and MS Data Obtained for the Carbohydrates Identified in Lager 2 by LC-NMR/MS

RT (min)	NMR[a]	MS (positive-ionzn) *m/z*	MS (positive-ionzn) molecular ion[b]	cpds identified (M_w)
13.5	low S/N	692	$[DP4 + Na]^+$	DP4 (M_w 666.6)
15.3	low S/N	530	$[DP5 + Na]^+$	DP3 (M_w 504.5)
17.3	S/N improved in loop spectrum	367	$[DP2 + Na]^+$	isomaltose and trehalose (M_w 342.3)
21.5	Glucose profile	204	$[hexose + Na]^+$	glucose (M_w 180.2)
23.0	Fructose profile	204	$[hexose + Na]^+$	fructose (M_w 180.2)

[a] S/N = signal-to-noise ratio. [b] DP = degree of polymerization.

well as of wort, to correlate the carbohydrate composition with the production process and to explore the sensitivity of the method to detect new carbohydrates. The application of rapid and informative methods in regard to carbohydrate composition of beer may be the basis of a useful tool for product development and quality control.

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