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Determination of Total Carbohydrate Content in Beer Using Its Pre-column Enzymatic Cleavage and HPLC-RI

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Abstract Beside ethanol, carbohydrates are the main source of total energy in beer. While analyses of fermentable carbohydrates are important from the technological point of view, the total content of carbohydrates is relevant in terms of nutrition. A high-performance liquid chromatography (HPLC) method with refractometric (RI) detection was developed for determination of total carbohydrate content in beer. Using enzymatic reaction with amyloglucosidase, the carbohydrates were cleaved to yield glucose and short glucose oligomers of less than 10 units, and separated on HPLC ion exchanger Rezex RSO-Oligosaccharide column in Ag^+ mode. Optimum parameters were established for the enzymatic sample treatment and for the HPLC separation of reaction products. Calibration curves of glucose, fructose, maltose and simultaneously analyzed glycerol ranged from 0.001 to 0.5 g/100 ml, correlation coefficients of all calibration curves were 0.9999. The instrumental limits of quantification were 0.001 g/100 ml and they were verified using repetitive injections, with coefficient of variation (CV) less than 10 % in five replicates. The method limit of quantification was 0.01 g/100 ml since it was necessary to dilute the beer samples before chromatographic analysis. Recovery of the method in non-alcoholic and alcoholic beer was 98.5 %, and 92.3 %, respectively. Finally, ten non-alcoholic and 15 alcoholic beers from Czech market were analyzed using the method, the average content of total carbohydrates in non-alcoholic and alcoholic beers being 4.21 and 3.70 g/100 ml, respectively. These results are in a good correlation with the real extract of beer, which is on average 4.58 and 4.27 g/100 ml.

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Introduction

Beer is an important source of chief nutritional compounds such as carbohydrates and proteins, carbohydrates being the main source of total energy of beer. In this context, beer has become a basic part of the diet in many cultures (Bamforth 2004).

The total content of carbohydrates in fermented beverages including beer is a very important parameter from nutritional viewpoint. EU regulation No. 1169/2011 of the European parliament and the Council defines inter alia Union rules on food labelling applicable to all foods. The development of a reproducible method for determining total carbohydrates and also glycerol content in beverages is therefore necessary. Although the development of modern chromatographic separation and detection techniques has considerably progressed, a routine method using these techniques for determining the total carbohydrate content in beer-based beverages has not yet been published. The final beer product contains fermentable carbohydrates, fructose, glucose and sugars with 2 and 3 degrees of polymerization (DP2 and DP3) and also oligomers with a higher level of polymerization (up to DP10) and polymers (above DP10). Usually, the fermentable carbohydrates are simply measured by an official EBC method with refractometric detection (RI) using Aminex column in Ca^{2+} form (EBC 9.27 2009). However, this method is not suitable for determination of carbohydrates with higher polymerization degree (DP4 –DP10). A similar method is prescribed by MEBAK analytica, where packed column Aminex HPX-87 and RI detector are used (MEBAK 2.7.2 2013). The total content of carbohydrates in beer is at present determined by

EBC (EBC 9.26 2009) and MEBAK (MEBAK 2.7.3 2013). Both of these methods, based on the hydrolysis and dehydration of carbohydrates with 85 % v/v sulfuric acid, result in the formation of 5-hydroxymethylfurfural, which reacts with anthrone to produce a blue-green color measured at 625 nm and the Regulation (EU) No. 900/2008 valid until 2008 (hydrolysis of dextrins using sodium hydroxide and subsequent spectrophotometric determination of glucose), though they exist, do not meet the requirements of the Regulation (EU) 117/2010, which requires the enzymatic conversion of polymers and oligomers of carbohydrates to glucose using amylase or amyloglucosidase followed by high-performance liquid chromatography (HPLC) determination.

Simple carbohydrates are also very often analyzed in beverages by amino bonded phases (Uchida et al. 1991). Modern amino columns make it possible to separate a wide range of sugar types including oligomers of carbohydrates; hence, a gradient mode of the mobile phase (water/acetonitrile) is necessary (Nogueira et al. 2005) and these columns therefore cannot be coupled with RI detection, which, as it is well known, are not compatible with gradient elution. Therefore, evaporative light scattering detection (ELSD), which is independent of the character of the mobile phase, is used in such cases. Floridi et al. (2001) used this technique for separating fermentable and non-fermentable carbohydrates of up to 15 glucose units. Estevinho et al. (2009) estimated the uncertainty of determination of lower concentrations (20–300 mg/l) glucose using ELSD in aqueous matrices. Further, carbohydrates with polymerization degree up to 6 and 7 were separated by gradient elution in beer samples in the application of Alltech Company (Alltech 2002). Finally, monosaccharides and malto-oligosaccharides were determined using simple gradient elution and ELSD in beer with different ethanol levels (Nogueira et al. 2005). The elution order of the reverse amino columns is determined by the size of the sugar molecules. The smallest molecules (fructose, glucose) elute first. Carbohydrates with larger molecules elute with increasing gradient.

The elution order of carbohydrate molecules on the polymeric columns with combined cation exchange and size exclusion principles is opposite to the elution on reverse columns (Phenomenex 2014). The molecules of oligomers with a higher degree of polymerization elute faster than molecules with a lower degree of polymerization. Separation on this column is performed in isocratic mode with water as the mobile phase and is coupled with RI detector.

The aim of this study was to develop a routine analytical method for determination of the total content of carbohydrates in beer with a broad range of alcohol contents (non-alcoholic beer and beer with various contents of alcohol). The principle of this method is conversion of polymers and oligomers of carbohydrates to glucose by enzymatic cleavage and determination of glucose using HPLC with RI detector on a column with combined cation exchange and size exclusion principles. Conditions of enzymatic cleavage were studied with regard to the inhibitory effect of alcohol on the enzymatic reaction in order to reach the best recovery of the cleavage products.

Materials and Methods

Chemicals

Standards of glucose (>99 %), fructose (>99 %) and maltose monohydrate (>99 %) were purchased from Merck (Czech Republic), glycerol (99 %) from Sigma Aldrich (Czech Republic). Maltodextrin standard GMD-6 was obtained from Dextra Laboratories (UK); it contained 65 % of DP1-DP12 glucose oligomers and 35 % of DP 13–DP 500 glucose polymers.

The enzyme amyloglucosidase (1,4-α-D-glucan glucohydrolase) from *Aspergillus niger* was obtained from Sigma Aldrich (Czech Republic) in the form of lyophilized powder. Phosphate buffer was prepared from 0.1 M KH_2PO_4 (p.a. Lach-Ner, CZ), final pH (4.8) was adjusted by phosphoric acid (85 %, p.a. Merck, CZ). Syringe filters with regenerated cellulose (Nalgene™, 0.45 μm) were purchased from SISW (Czech Republic).

Beer Samples

Non-alcoholic beer with original gravity of 5.01 % (w/w) and alcohol content of 0.4 % (v/v) was used for the development of the method. This beer with an addition of maltodextrin (2 or 4 g of maltodextrin in100 ml of non-alcoholic beer) and ethanol (5 % and 8 % v/v into non-alcoholic beer) was used for developing the method for various types of alcoholic beer and for assessing the method recovery and robustness. The relevance of the results was ensured by volume correction, the samples without addition of ethanol were doped with an adequate volume of water.

Real samples of ten non-alcoholic beers and 15 lager beers were used for method verification. These beers were obtained from the Czech market.

Model Samples

Model samples of beer were used during the method development because of its defined composition in contrast to beer matrix, which can change from brand to brand. Moreover, we needed samples with exactly defined total carbohydrate content. Therefore, a model sample of non-alcoholic beer was prepared as a solution of 4 g of maltodextrin dissolved in 100 ml of 0.1 M phosphate buffer, pH 4.8. A model sample of the alcoholic beer was prepared as a solution of 4 g of maltodextrin dissolved in 100 ml of 0.1 M phosphate buffer, pH 4.8 and ethanol (5 % or 8 % v/v).

Enzymatic Cleavage

Enzyme solution was prepared by dissolving 0.12 g of amyloglucosidase in 10 ml of deionized water. A 25-ml sample (degassed and filtered beer or model sample) was tempered for 15 min at 60 °C. Then 1.2 ml of enzyme solution was added to the sample and the mixture was incubated for 120 min at 60 °C. The enzyme was inactivated by a 15-min incubation of the mixture at 85 °C. Finally, the sample was cooled with water at a laboratory temperature and centrifuged for 15 min at 10,000 rpm and 20 °C. After centrifugation, the sample was diluted ten times, filtered through a syringe filter and then analyzed by HPLC-RI.

The volume of blank sample (without enzyme) was corrected by adding 1.2 ml water into 25 ml of sample.

Analytical Method

Apparatus

HPLC-RI analysis was carried out on a high-pressure pump with degasser, column thermostat (SISw, Czech Republic) and autosampler Midas (Spark, Holland) connected with a high-sensitivity RI detector Shodex RI 101 (Japan). Chromatographic data were collected and processed by the DataApex Clarity data system, version 3.0.5.505.

Chromatographic Conditions

Separations were performed on Rezex RSO-Oligosaccharide ion exchange column in Ag^{+} mode (200×10 mm; Phenomenex, USA) with deionized water as mobile phase (Millipore S.A., France). The flow rate was 0.3 ml/min, and the column temperature was 80 °C.

During the method development, we also used an IEX Ca^{2+} (250×8 mm; Watrex, Prague) polymer column in isocratic mode with deionized water (Millipore S.A., France) as the mobile phase; the flow rate was 0.5 ml/min, and the column temperature 90 °C.

The injection volume on both columns was 10 μl.

Calibration Curve

The calibration curve was prepared over a linear range at five calibration levels, from 0.001 to 0.5 g/100 ml, where the calibration solution at every level was formed by a mixture of glucose, fructose, maltose monohydrate and glycerol. The concentration levels of standard solution were 0.001, 0.01, 0.02, 0.1, and 0.5 g/100 ml for each standard. A new calibration curve was constructed before each series of measurements.

Real Extract Measurement

The total carbohydrate content in beer represents the majority of real extract. A minor portion of the extract is present in the form of proteins, glycerol, beta-glucans, pentosans, minerals, organic acids and other compounds (Briggs et al. 2004). Therefore, the values of real extract were used as control method during the study. Real extract measurement was performed on Anton Paar DMA 4500 densitometer (Anton Paar, Austria) according to the EBC 9.4 (2009) method; real extracts were expressed in % (w/w).

Results

The recovery of the products of enzymatic cleavage reaction and their high performance chromatographic separation were key parameters of the final method. Since the recovery of the reaction products is influenced by the alcohol content of analyzed beer, the method was optimized for non-alcoholic beer, common alcoholic beer and also for beer with higher content of alcohol.

Table 1 Optimization of conditions of enzymatic reaction

Conditions of enzymatic reaction			Concentration of resulting carbohydrates (g/100 ml)			
Time (min)	Temperature (°C)	Concentration of enzyme (g/10 ml)	Maltose[a]	Glucose[a]	Fructose[a]	Sum
60	50	0.24	0.16	3.02	0.03	3.21
45	60	0.24	0.16	3.01	0.04	3.21
30	65	0.24	0.15	2.78	0.04	2.97
60	65	0.24	0.14	2.96	0.04	3.14
60	55	0.24	0.14	3.15	0.01	3.30
120	55	0.12	0.15	3.15	0.01	3.31
60	60	0.24	0.14	3.17	0.02	3.33
120	60	0.12	0.14	3.16	0.01	3.31

Determination of cleavage products on Ca^{2+} column

[a] Average of three measurements

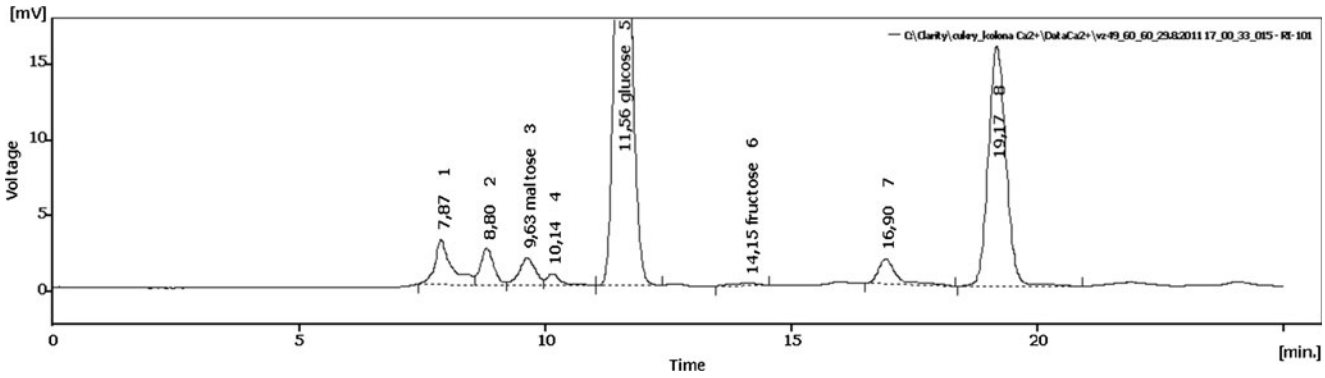

Fig. 1 Chromatogram of products of enzymatic reaction in beer. Separation on ionex column in Ca^{2+} cycle. maltose 9.63 min, glucose 11.56 min, fructose 14.15 min, ethanol 19.17 min; other not-well separated zones and also the frontal peaks are non-separated compounds. For chromatographic conditions, see Materials and methods

Optimization of Enzymatic Reaction and HPLC Conditions

We first studied the enzymatic cleavage of carbohydrates in non-alcoholic beer and also in a model sample (0.1 M phosphate buffer, pH 4.8). The subsequent studies were performed with both these matrices supplemented with 5 % and 8 % (v/v) of ethanol, respectively. Simultaneously, conditions for the efficient chromatographic determination of the ensuing simple sugars and short-chain oligomers were optimized. Finally, the developed method was tested on real beers and the resulting total carbohydrate content so ascertained was compared with the real beer extracts (%, w/w) measured by a routine densitometry method on Anton Paar beer analyzer (EBC 9.4 2009). Final optimization of the method was performed based on the correlation of the two sets of data.

The supplier of amyloglucosidase (from *A. niger*) declares the temperature optimum of the enzymatic reaction at 50 °C. However, we observed that higher temperature positively influences the reaction rate and decided to perform test trials, with the enzymatic reaction running at 50 °C, 60 °C and 65 °C and with 30, 45 and 60 min reaction time. The concentration of the enzyme in the reaction was 0.24 g per 10 ml of tested beer in all trials. The highest recovery of the reaction products was achieved at 60 °C and 60-min reaction time. To reduce the relatively high consumption of the enzyme in the reaction, we finally used a half concentration of the enzyme and 120-min reaction time; the recoveries under these conditions were the same (Table 1). Unfortunately, when we analyzed the products of the enzymatic reaction in beer on Ca^{2+} column using the recommended EBC method (EBC 9.26 2009), the chromatogram (see Fig. 1) featured, besides glucose and fructose, also a peak of maltose, some incompletely separated zones and also the frontal peaks of non-separated compounds, which probably led to the large error of quantification. The final concentration of glucose (3.17 g/100 ml) in non-alcoholic beer obtained under these conditions did not respond to the value of real extract, which was 5.01 % w/w. Also the concentration of total carbohydrates in the model sample was much lower

Table 2 The comparison of carbohydrate oligomers profile and total amount of carbohydrates in both, original and cleavaged sample, and ethanolic and non-ethanolic model sample

Ethanol addition	0 %		5 %	
	Original (g/100 ml)[a]	Cleaved (g/100 ml)[a]	Original (g/100 ml)[a]	Cleaved (g/100 ml)[a]
Glucose	0.04	3.74	0.04	3.33
Maltose	0.20	0.03	0.20	0.07
DP3	0.31	0.01	0.31	0.01
DP4	0.19	<0.01	0.19	0.01
DP5	0.18	0.01	0.18	0.02
DP6	0.36	0.01	0.36	0.01
DP7	0.32	0.01	0.32	0.01
DP8	0.10	0.01	0.10	0.01
DP9	0.04	<0.01	0.04	0.01
DP10	0.02	<0.01	0.02	<0.01
Sum	1.76	3.83	1.76	3.48
Recovery (%)	44.0	95.5	44.0	87.0

HPLC determination on Ag^{2+} column

[a] Average of three measurements

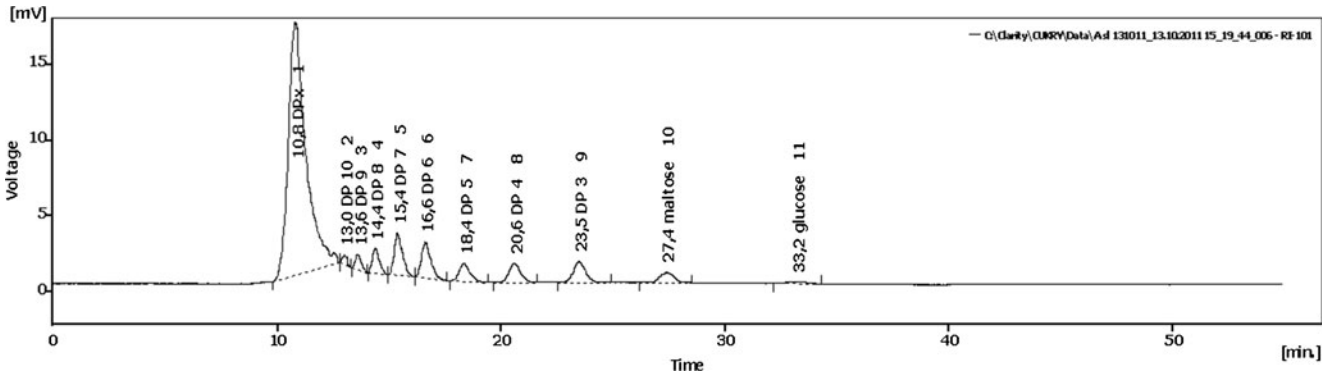

Fig. 2 Chromatogram of carbohydrate profile before enzymatic cleavage in maltodextrin model sample. Separation on ionex column in Ag^+ cycle under final conditions. DPx, 10.9 min; DP3–DP10, 12.9–23.1 min; maltose, 27.6 min; glucose, 33.2 min; frontal peak of non-separated compounds. For chromatographic conditions, see Materials and methods

(2.56 g/100 ml) than the reference value (4.0 g/100 ml). As noted below, the low yield was caused by a low efficiency of the chromatographic system and the method was therefore further optimized.

Because a close to 100 % transformation of polymers and oligomers of carbohydrates to glucose during the optimization of the enzymatic reaction was not achieved, we decided to quantify the total carbohydrate content of the products as a sum of glucose and higher oligomers with up to DP 10 using a more efficient chromatographic system. Since simple carbohydrates and their oligomers formed by enzymatic cleavage of beer were not separated on the Ca^{2+} column with good efficiency, we used a cation exchange polymeric column in Ag^+ cycle, which is able to separate oligomers of glucose with the degree of polymerization ranging from 2 to 10 (DP2–DP10) and sometimes over DP 10. The recovery attained by the method was improved and it is described below in detail. In addition, under the improved chromatographic conditions glycerol is well separated from the sugars and can thus be taken as an additional analyte readily identified by this method. The concentration of glycerol is used when determining the energetic value of beer, which is calculated as the sum of contributions of carbohydrates, glycerol, alcohol, and total peptides (Regulation 1169/2011).

Influence of Ethanol on Enzymatic Reaction

Model Sample

Maltodextrin GMD-6 is constituted by polymers of glucose units; it should be equivalent to the complex of the majority of carbohydrates of beer. Therefore, maltodextrin was used for preparing the model sample as a standard of "carbohydrates" in beer for assessment of method repeatability and recovery. Furthermore, the model sample could be used for the control of enzymatic activity of stored enzyme or when a new lot of enzyme is used.

The recovery of the enzymatic reaction was assessed in the model sample. The tested maltodextrin concentration of 4 g/100 ml corresponds approximately to the total amount of carbohydrates in beer (with an original gravity of 12 %). Two variants were tested; model sample without ethanol represents non-alcoholic beer, model sample with an addition of 5 % (v/v) of ethanol represents alcoholic beer. Comparison of the total

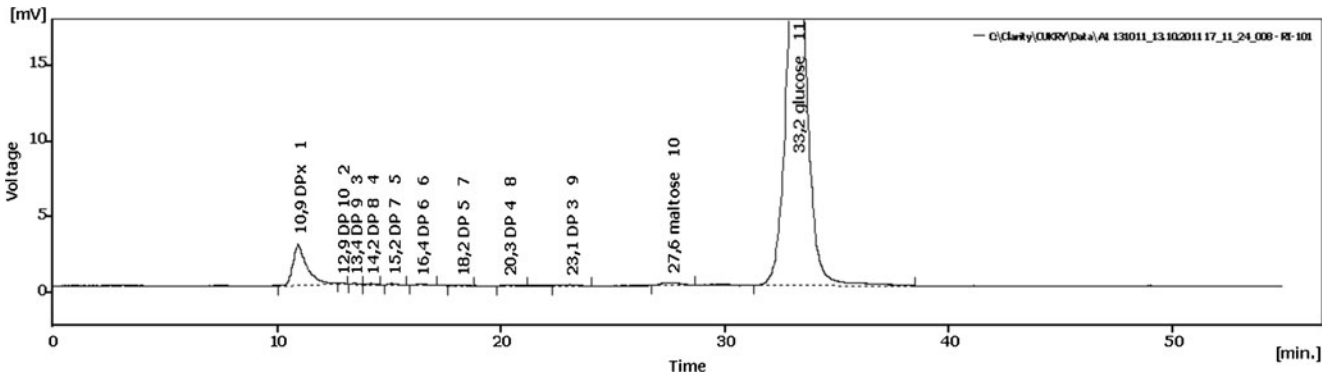

Fig. 3 Chromatogram of carbohydrate profile after enzymatic cleavage in model sample. Separation on ionex column in Ag^+ cycle under final conditions. DP3–DP10, 11.9–23.1 min; maltose, 27.5 min; glucose, 33.1 min; glycerol, 37.1 min; ethanol, 40.6 min. For chromatographic conditions, see Materials and methods

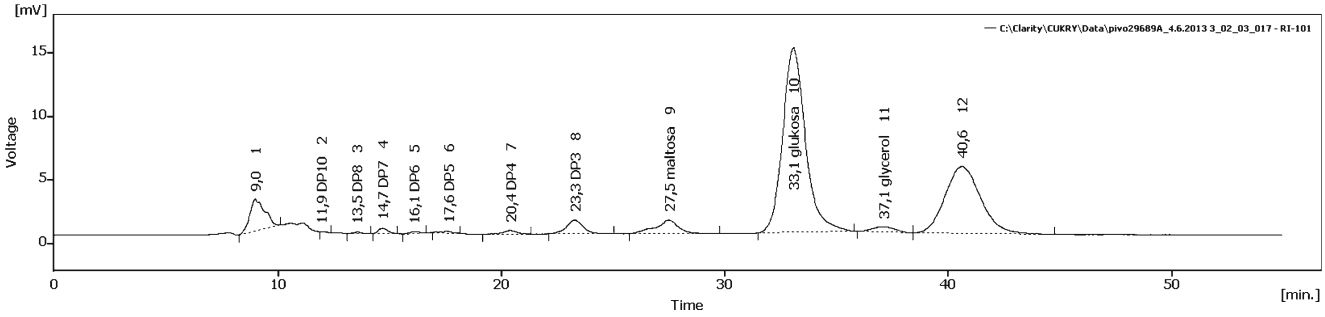

Fig. 4 Chromatogram of carbohydrate profile after enzymatic cleavage in real beer. Separation on ionex column in Ag^+ cycle under final conditions

amount of carbohydrates before and after cleavage in ethanolic and non-ethanolic model sample is presented in Table 2. The concentration of each of the DP3–DP10 oligomers is also presented; each oligomer was corrected using anhydrous maltose factor (0.950002). The chromatograms of beer before and after cleavage are presented in Figs. 2 and 3. The resulting oligomers of up to DP10 were separated with good efficiency on an ion exchange column in Ag^+ cycle. Non-separated oligomers and/or polymers elute in the first zone and were marked as DPx. The shape of this zone is often asymmetrical, especially in the real samples. However, the concentration of DPx is so negligible that it can be ignored. The total amount of carbohydrates in beer can then be calculated from the formula:

$$c_{\mathrm{tot}} = c_{\mathrm{glu}} + c_{\mathrm{mal}} + \sum_{y=3}^{10} c_{\mathrm{DP}y},$$

where c_{tot} is total carbohydrate content, c_{glu} is concentration of glucose, c_{mal} is concentration of maltose, and $c_{\mathrm{DP}y}$ are concentrations of non-cleaved glucose oligomers with the degree of polymerization from 3 to 10.

As follows from Table 2, the total original concentration of carbohydrates in both alcoholic and non-alcoholic model samples before enzymatic cleavage is absolutely the same (1.76 g/100 ml). The original concentration of maltodextrin model sample was again 4 g/100 ml. As expected, differences were found between alcoholic and non-alcoholic samples after cleavage. The concentration of glucose formed in the alcoholic samples was only 3.43 g/100 ml while in non-alcoholic samples it was 3.82 g/100 ml. Also the concentration of maltose was higher in non-alcoholic samples. The presence of ethanol thus partially lowers the activity of the enzyme, which results in the reduction of the yield of glucose and other low glucose oligomers. To sum up, the calculated efficiency of the enzymatic cleavage was 95.5 % and 87.0 % for non-alcoholic and alcoholic samples, respectively (see Table 2).

Beer Sample

Analogous experiments were performed with beer matrix, namely with non-alcoholic beer with or without an addition of ethanol. The chromatogram of carbohydrate profile after enzymatic cleavage in real beer is shown in Fig. 4. A good resolution of separated peaks of DP3–DP10 oligomers, maltose, glucose, glycerol and ethanol was obtained. The results are presented in Table 3. Surprisingly, the inhibitory influence of ethanol (ca. 5 % v/v) on enzymatic cleavage in beer matrix was not as high as in the model sample. As shown in Table 3, the concentrations of glucose and oligomers and their sum after conversion in both beer samples are comparable — it was 4.08 and 3.87 g/100 ml in the non-alcoholic and alcoholic, respectively.

Method Validation

Calibration Curve, Linearity, Limit of Quantification

The calibration curves of glucose, fructose, maltose monohydrate, and glycerol were prepared over a linear range from 0.001 to 0.5 g/100 ml. The resulting regression equations

Table 3 The comparison of carbohydrate oligomers profile and total amount of carbohydrates in both, original and cleavaged, and ethanolic and non-ethanolic beer samples

Original samples			Cleaved samples	
Ethanol addition	0 % (g/100 ml)[a]	5 % (g/100 ml)[a]	0 % (g/100 ml)[a]	5 % (g/100 ml)
Fructose	0.04	0.04	0.06	0.04
Glucose	0.01	0.01	3.84	3.58
Maltose	1.97	1.97	0.10	0.14
DP3	0.61	0.61	0.04	0.06
DP4	0.19	0.19	0.01	0.02
DP5	0.06	0.06	<0.01	<0.01
DP6	0.06	0.06	<0.01	<0.01
DP7	0.04	0.05	<0.01	<0.01
DP8	0.04	0.04	<0.01	<0.01
DP9	0.03	0.02	<0.01	<0.01
DP10	0.01	0.01	<0.01	<0.01
Sum	3.07	3.05	4.08	3.87

HPLC determination on Ag^{2+} column

[a] Average of three measurements

Table 4 Validation parameters of the method

Parameter (g/100 ml)	Glucose	Fructose	Maltose	DP4–DP10	Glycerol	Total carbohydrates
k[a]	1,952.1564	2,114.88729	3,950.93437	–	1,225.69108	–
q[b]	2.1861	−0.2347	−2.11261	–	0.93	–
R***	0.9999	0.9999	0.9999	–	0.9999	–
ILOQ	0.001	0.001	0.001	0.001	0.001	–
CV (%)	7.04	9.09	10.3	–	9.61	–
MLOQ	0.01	0.01	0.01	0.01	0.01	–
r_{95}	0.15	0.02	0.04	0.02	0.01	0.2

[a] Slope of calibration curve ($y=kx+q$)

[b] Intercept of calibration curve

[c] correlation coefficient

are shown in Table 4. The linearity of all calibration curves in this concentration range was determined; predictions of linearity were made on the basis of the fitted curve for linear and quadratic regression models (Loco et al. 2002). Correlation coefficient for all regressions reached the value of 0.9999 (see Table 4).

The instrument limit of quantification (ILOQ) was determined as the lowest point of the calibration curves of glucose, fructose, maltose and DP4–DP10 with a coefficient of variation (CV) less than 20 % in five replicates (see Table 4). The real values of CV were evaluated as still lower, specifically below 10 %. Because the concentration of carbohydrates is ten times higher than the range of the calibration curve, the beer was diluted ten times before the measurement. Therefore, the method limit of quantification (MLOQ) was determined as the lowest amount of carbohydrates that can be quantified in a diluted sample of analyzed beer; ILOQ was multiplied by the dilution factor of 10.

Repeatability

Finally, repeatability r_{95} of the method was determined as 2.8-fold the summary standard deviation, calculated from the parallel determination of 15 samples of beer by the formula

$$\mathrm{SOD}_{\mathrm{sum}} = \sqrt{\left[\sum \left(x_i - x_j\right)^2 / 2n\right]},$$

where x_i, and x_j are two parallel estimations and n is the number of samples. Repeatability r_{95} of glucose, fructose, maltose, sum of DP3—DP10, glycerol and total carbohydrate

Table 5 Recovery of the method

Ethanol addition (% v/v)	0 %			5 %			8 %	
Maltodextrin addition (g)	0 (g/100 ml)[a]	2 (g/100 ml)[a]	4 (g/100 ml)[a]	0 (g/100 ml)[a]	2 (g/100 ml)[a]	4 (g/100 ml)[a]	0 (g/100 ml)[a]	4 (g/100 ml)[a]
Fructose	0.06	<0.01	<0.01	0.07	<0.01	<0.01	0.06	<0.01
Glucose	3.84	5.80	7.72	3.85	5.68	7.55	3.71	7.29
Maltose	0.10	0.15	0.15	0.11	0.14	0.15	0.10	0.16
DP3	0.04	0.04	0.05	0.05	0.04	0.05	0.04	0.07
DP4	0.01	<0.01	0.02	0.02	0.01	0.02	0.01	0.03
DP5	<0.01	<0.01	0.01	<0.01	<0.01	0.01	<0.01	0.02
DP6	<0.01	<0.01	0.01	<0.01	<0.01	0.02	<0.01	0.02
DP7	<0.01	<0.01	0.02	<0.01	0.02	0.03	<0.01	0.04
DP8	<0.01	<0.01	0.04	<0.01	<0.01	0.01	<0.01	0.02
DP9	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	0.01
DP10	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Sum	4.07	6.03	8.03	4.13	5.94	7.85	3.95	7.67
Determined addition (g/100 ml)		1.96	3.96		1.81	3.72		3.72
Recovery (%)		98.0	99.0		90.5	93.0		93.0

HPLC determination on Ag^{2+} column

[a] Average of three measurements

content was 0.15, 0.02, 0.04, 0.02, and 0.2, respectively (shown in Table 4).

Recovery, Robustness

The recovery of the method was determined for common beer with alcohol content ranging from 0 % to 8 % (v/v) and original gravity ranging from 4 % to 20 % (w/w). This experiment was also performed to demonstrate good robustness of the method. Therefore, non-alcohol beer was used with an addition of 2 and 4 g of maltodextrin to 100-ml beer samples and also with an addition of 5 % and 8 % of ethanol. Resulting recoveries for all types of tested beer are given in Table 5. The recovery of the method for non-alcoholic beer and beer with 5 % and 8 % of ethanol is 98.5 %, 91.1 % and 92.3 %, respectively. The conditions of the enzymatic reaction are sufficient to cleave a higher concentration of carbohydrates with the same recovery as in standard beer, which verifies the good robustness of the method.

Verification and Application of the Method

Because it is impossible to gauge the exact composition of beer, we used the comparison of the total carbohydrate amount with the real extract of beer for verification of our results. Furthermore, actual information about this ratio is not available in the literature. Therefore, we compared the real extract with total carbohydrate content in 15 alcoholic and ten non-alcoholic real beer samples. The contents of total carbohydrates in alcoholic beers and real extract were ranging from 2.85 to 4.35 g/100 ml and from 3.4 % to 5.0 %, respectively (see Table 6). This means that carbohydrates represented 80.3–93.5 % of real extract, the mean value being 86.5 %. The content of total carbohydrates and real extract of non-

Table 6 Total carbohydrate content versus real extract in analyzed beers

Sample	Real extract value (% w/w)	Carbohydrate content (g/100 ml)	Abs. difference (g/100 ml)	Rel. difference (%)	Percentage of carbohydrates in extract (%)
Nonalcoholic					
Beer 1	3.17	3.07	0.10	3.2	96.8
Beer 2	3.31	3.24	0.07	2.1	97.9
Beer 3	4.58	4.59	−0.01	−0.2	100.2
Beer 4	4.46	4.31	0.15	3.4	96.6
Beer 5	5.01	4.70	0.31	6.3	93.7
Beer 6	3.70	3.43	0.27	7.3	92.7
Beer 7	4.80	4.48	0.32	6.8	93.2
Beer 8	3.33	3.05	0.28	8.3	91.7
Beer 9	7.97	8.08	−0.11	−1.4	101.4
Beer 10	3.49	3.16	0.33	9.5	90.5
Mean	4.27	3.70		4.5	95.5
Alcoholic					
Beer 1	4.28	3.94	0.34	7.9	92.1
Beer 2	3.44	3.19	0.25	7.3	92.7
Beer 3	4.55	4.03	0.52	11.4	88.6
Beer 4	4.17	3.70	0.47	11.3	88.7
Beer 5	4.44	3.68	0.76	17.1	82.9
Beer 6	4.09	3.55	0.54	13.2	86.8
Beer 7	4.26	3.74	0.52	12.2	87.8
Beer 8	5.01	4.33	0.68	13.6	86.4
Beer 9	5.02	4.35	0.67	13.3	86.7
Beer 10	4.10	3.38	0.72	17.6	82.4
Beer 11	3.53	2.85	0.68	19.3	80.7
Beer 12	4.59	3.97	0.62	13.5	86.5
Beer 13	4.22	3.39	0.83	19.7	80.3
Beer 14	4.31	3.74	0.57	13.2	86.8
Beer 15	4.13	3.62	0.51	12.3	87.7
Mean	4.58	4.21		13.5	86.5

alcoholic beers was ranging from 3.07 to 8.08 % and from 3.2 to 7.9 %, respectively. In this case, carbohydrates represented 90.5–100 % of real extract, with a mean value of 95.5 %. The higher percentage of carbohydrates in the real extract of non-alcoholic beer is logical, because this beer is less fermented and, therefore, the per cent content of the other compounds is lower than in common alcoholic beer. These close correlations verified the good accuracy of our results and the method.

Discussion

Although according to some authors the refractometric detector has many disadvantages, such as low sensitivity or incompatibility with gradient elution (Nogueira et al. 2005), it follows from our data that the RI detector permits the determination of carbohydrate concentrations as low as 0.001 g/100 ml. It is therefore suitable not only for determining total carbohydrate content but also for determination of the carbohydrate profile. Its sensitivity is so high that the sample of beer has to be diluted ten times before the measurement. Further, the incompatibility with gradient elution can be solved by using a highly effective column working in isocratic mode; these columns are already available on the market.

Brewery and food laboratories which possess RI detectors and use the EBC 9.27 and MEBAK 2.7.2 methods for direct determination of fermentable sugars (glucose, fructose, maltose and maltotriose) can use our new method for determination of total carbohydrate content. They are likely to use currently the official EBC 9.26 and MEBAK 2.7.3 methods; however, these methods do not comply with current legislation (EU Regulation 117/2010), which requires analytical HPLC determination. Though an enzymatic reaction does not have a hundred percent efficiency, our study has proved that enzymatic cleavage combined with HPLC determination is a satisfactory method with good recovery and repeatability. Residual oligomers (DP3–DP10), which are added to the major peak of glucose, are probably short glucose chains with a non-alpha side bond. Finally, the concentration of DPx is so negligible that it can be ignored.

It is interesting to compare the total carbohydrate content with the sum of glucose and its oligomers before enzymatic cleavage. It is evident that nearly 50 % of carbohydrates are presented in beer in the form of chains longer than 10 glucose units. When these carbohydrates with a high degree of polymerization do not undergo cleavage, they are not retained on the column and elute with the frontal peak.

Determination of total carbohydrates in model sample should be regularly used (over a 2-month period) to control the enzymatic activity of stored or newly purchased enzyme. Long-term stability tests of the enzyme indicate that the enzyme used in our study is stable, and every batch of the enzyme has the same properties.

The results demonstrate that the total amount of carbohydrates in beer could be determined even if the transformation of carbohydrates into glucose is not complete. The sum of glucose and partially formed oligomers provides a good recovery.

Conclusion

Determination of total carbohydrate content in beer using enzymatic cleavage of poly- and oligomers of glucose and a follow-up HPLC-RI method represents a simple way how to express the total carbohydrate content as an equivalent of glucose. The major product of enzymatic cleavage is glucose. Although the enzymatic conversion is not hundred per cent, an accurate result can be obtained because the resulting carbohydrate oligomers are determined together with glucose as the main product using a highly efficient chromatographic method after the termination of enzymatic cleavage. The method is suitable for determination of carbohydrates in various types of beer-based beverages. When the concentration of fructose and glycerol is to be assayed, it is better to determine it by HPLC-RI before carbohydrate conversion, because a huge peak of the glucose formed can interfere with the concurrently eluting peak of fructose and glycerol, which make the quantification of these minor peaks impossible.

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Compliance with Ethics Requirements This article does not contain any studies with human or animal subjects.

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