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Carbohydrate determination in wort and beer by HPLC-ELSD

Complete separation of fermentable and non fermentable carbohydrates of up to 15 glucose units in wort, mashed malt and beer was obtained in 40 minutes by direct injection on a NH_2 column and an HPLC-ELSD apparatus. The results were comparable with those found with the official HPLC-RI procedure and with chemical determination with Fehling's reagent. In Italian wort and mashed malt, about 88% of the total carbohydrates are fermentable, while in beer only 29% are fermentable.

BC 35 Wort

(Descriptors: Carbohydrates, wort, beer, oligosaccharides, HPLC-ELSD.

Deskriptoren: Kohlenhydrate, Würze, Bier, Oligosaccharide, HPLC-ELSD).

1 Introduction

Carbohydrates are important substances in the brewing process. They are responsible for the alcohol content, taste, flavor, color, carbonization, foam production, and nutritional quality of beer [1] [2]. Therefore, knowledge about their determination is important for the brewer and for beer quality assessment [3] [4]. The amount of carbohydrates during the brewing process depends on barley type/cultivar or other cereals, malting and brewing conditions, yeast strains, etc.

Analysis of the sugar in mashed malt and wort allows the processes of malting and mashing to be monitored, while determination of fermentable and non fermentable sugars in beer gives information about the fermentation process, yeast performance, and the organoleptic quality of beer.

Glucose, fructose, sucrose, and sugars with a high Degree of Polymerization (DP), such as maltose (DP2) and maltotriose (DP3), are the main fermentable carbohydrates in malt and wort, while non-fermentable polymeric carbohydrates occur in minor amounts. On the other hand, the latter predominate in beer and include maltotetraose (DP4) and other maltose polymers (DPn) containing units up to 10 α-(1$\rightarrow$ 4) bonded units (oligosaccharides); higher polymers reaching up to 20 glucose units (megalosaccharides) also occur. Non fermentable sugars contribute to the colloidal stability of beer and are recognized for being responsible for taste smoothness [1]. Pentoses occur in beer but in trace amounts and contribute little to beer quality [5].

Great importance is given to determining the carbohydrate fractions or individual carbohydrates when adjuncts are added to the brewing process [6].

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Generally, carbohydrates are commonly separated by HPLC using both polymeric ion exchange or amino bonded columns with isocratic aqueous or gradient organic eluents [7]. However, there may be major differences in their detection. UV detection involves the use of low range wavelength (<210 nm) which is not selective for sugars. Refractive Index (RI) appears to be the most widely used detector for sugars; low sensitivity and selectivity are its main constraints, and gradient elution can not be used [7]. Recently, post-column derivatization [8] or Electrochemical Detection (ECD) [9-11] has been introduced to improve sensitivity but only low DP sugars are able to be determined.

Evaporative Light Scattering Detection (ELSD) has been successfully introduced in carbohydrate measurement in foods and beverages [8] [12–16]. The advantage of ELSD is its high sensitivity, good reproducibility and low signal to noise ratio. Moreover it allows gradient elution to be used, which is essential when a wide range of polymeric forms of carbohydrate has to be separated [7].

Carbohydrates in mashed malt, wort and beer are usually determined by chemical and HPLC methods. Total reducing carbohydrates (RS) are determined by the oxidoreducing Fehling's reagent, while colorimetry using anthrone and other chromogens is used for total carbohydrates (TC) [1].

Individual carbohydrates are determined by HPLC. The official methods use polymeric ion columns to separate only the main fermentable sugars: fructose, sucrose, glucose, maltose and maltotriose [17] [18]. The disadvantages of these methods are the requirement of mineral acids as eluent, RI detection, incomplete peak resolution, high temperature of separation, and time consuming sample preparation because of the previous deionization by ion exchange resin [17] [18].

No official methods are available to determine higher individual sugars. Only acid or enzymatic hydrolysis is available to determine their glucose equivalent [1].

Several researchers have proposed methods to improve the analysis of individual carbohydrates in wort and beer. *Gotschik* and *Benson* [19] and *Bernal* et al. [20] were able to separate only simple sugars occurring in beer by HPLC. *Donhauser* and *Wagner* [21] obtained similar results with an isocratic elution in an amino bonded column by direct injection of the sample.

Comparison between ELSD and RI resulted in non significant differences between the two detectors [14] [16] in the determination of fermentable carbohydrates.

Uchida et al. [22] were the first to separate oligosaccharides up to DP10 in less than 30 min by HPLC in beer and wort. *Lehtonen* and *Hurme* [15] separated and quantitated mono- and oligosaccharides up to DP7 in Finnish beers using an ELSD detector. Recently *Shan* et al. [23] proposed an HPLC procedure to evaluate fermentable carbohydrates up to maltotriose on a NH_2 column and RI detection.

The aim of the present research was to develop a method to separate all individual fermentable carbohydrates occurring in wort and beer, and to separate and evaluate quantitatively the individual oligomeric carbohydrates up to 15 maltose units.

Good reproducibility and sensitivity for all carbohydrates was obtained in 40 minutes, using an amino-bonded silica column, aqueous/acetonitrile gradient elution profile at room temperature, an ELSD detector, a simple sample preparation, in good agreement with the determination by Fehling's reagent.

The official method by HPLC-RI was used as control.

2 Materials and methods

2.1 Samples and reagents

Samples of mashed malt, wort and beer were used. Mashed malt, wort and beer samples were furnished by some Italian brewing factories. NaN_3 was added to the wort (100 mg/l) to prevent microbial growth after sampling and beer samples were tunnel pasteurized. Mashed malt was frozen (–18°C) and quickly thawed, while wort and beer samples were refrigerated (4°C) and stored until analysis.

Fructose and glucose were purchased from Carlo Erba (Milano, Italy), sucrose from Riedel-de-Haën (Hannover, Germany), maltose from Difco (Detroit, MI, USA), and maltotriose (DP3), maltotetraose (DP4), maltopentaose (DP5), maltohexaose (DP6) and maltoheptaose (DP7) from Sigma (Milano, Italy). HPLC grade acetonitrile and water were from Carlo Erba (Milano, Italy) and Panreac (Barcelona, Spain), respectively. Analytical grade nitrogen was from Linde Gas Tecnici (Perugia, Italy). All other reagents were analytical grade.

2.2 Sample preparation

The liquid portion of the mashed malt and wort samples were centrifuged and then diluted 1:5 with distilled water; 2-octanol (0.1% v/v) was added to beer samples to avoid foaming; samples were degassed by shaking, then centrifuged and filtered through a 0.4μm filter.

2.3 Calibration

A stock standard solution was prepared by weighing approx. 25~50 mg of the nine pure compounds in a flask and brought to a final volume of 50 ml with distilled water. NaN_3 (100mg/l) was added to prevent microbial growth. The actual amount of each compound was normalized according to the purity and hydration degrees reported on the label.

The final concentration of standard sugars was 0.5 – 1.0 g/l. The stock solution was stored at 4°C and prepared monthly.

The stock solution was diluted 1:2, 1:3 and 1:4, and utilized directly for HPLC analysis in order to evaluate recoveries.

2.4 Equipment and Conditions

A polymeric amino column Shodex-NH_2P-50, 4.6×250 mm, (Shodex Inc., Tokyo, Japan) and a bulk guard column containing the same support were used.

A Jasco PU-1580 pump, a Jasco ternary gradient mixer LG-1580-02 and a Jasco 3-line degasser DG-1580-53 (Jasco Corporation, Tokyo, Japan) performed the gradient by a low pressure mixing of the two solvents (acetonitrile/water).

A 7125 Rheodyne injection valve (Rheodyne Inc, Cotati, CA, USA) was utilized with a 10 μl loop. The run of the analysis was 40 min. All samples were filtered before injection using 0.2 μm pore size disposable nylon filters (Whatman, Maidstone, England, UK).

An Alltech ELSD-500, ELSD detector was used (Alltech Associates, Inc., Deerfield, IL, USA). Drift tube temperature was set at 110°C, nitrogen flow was set at 2.75 l/min.

The eluent flow rate was 1 ml/min at room temperature. The eluent mixture was maintained at 75% (v/v) acetonitrile in water for the first 10 min after injection, then programmed to 50% acetonitrile over a period of 15 min, and finally maintained at this concentration for 5 min. To recondition the whole system, the initial conditions were restored for 5 min.

A Varian IR-3 differential RI detector was used. The instrument was set a $10*10^{-6}$ Δ RI, the cell was maintained at room temperature. The chromatographic separation of fructose, glucose, sucrose, maltose and maltotriose was obtained by an isocratic mixture of acetonitrile/water 72:28 (v:v) from 0 to 20 min. To clean and to equilibrate to initial conditions, 50% of acetonitrile/water for 5 min and 72% acetonitrile/water for 5 min was used. The stock standard solution prepared as for HPLC-ELSD calibration contained 10÷100 g/l of sugars and NaN_3 (100mg/l); two dilutions were made (1:2 and 1:4).

2.5 Chemical analysis

The content of free reducing sugars in mashed malt, wort and beer was determined by a direct titration with Fehling's reagent according to the procedure of Lane and Eynon [1] and the results are reported as RS. After acid hydrolysis the TC content was determined and is reported as glucose equivalent units [1] [24].

Liquid portions of mashed malt and worts were centrifuged and diluted 1:10 before titration; beers were degassed. All prepared samples were then treated with activated charcoal (1% w/w) and filtered in order to remove phenolic compounds interfering with the determination [1].

5 ml of Fehling's reagent A were mixed with 5 ml of reagent B in a flask and diluted with distilled water to approx. 50 ml. The boiling Fehling's reagent was titrated with the sample solution until reaching the endpoint evaluated by adding 2 drops of 1% (w/v) aqueous methylene blue as indicator. The titration procedure was followed strictly because any variation may affect results [1].

Samples were then hydrolyzed with 1M HCl for 30 min at 80°C. to convert oligosaccharides to glucose, and then Fehling's reagent was titrated with the hydrolyzed sample to determine the TC content.

2.6 Statistical analysis

The t-paired test was performed to compare differences between the sets of data.

Table 1 Calibration coefficients of carbohydrates with ELSD and RI detection

	ELSD Area/1000 vs conc g/l			RI Area/1000 vs conc g/l		
compound	**slope**	**intercept**	**R^2**	**slope**	**intercept**	**R^2**
fructose	1928	441	0.9942	152	29	0.9997
glucose	1428	195	0.9974	13	38	0.9980
sucrose	2445	197	0.9994	46	27	0.9999
maltose	1303	210	0.9997	42	168	0.9995
maltotriose	1325	93	0.9972	40	47	0.9969
maltotetraose	1142	48	0.9948	/	/	/
maltopentaose	1032	13	0.9971	/	/	/
maltohexaose	1022	22	0.9959	/	/	/
maltohexaose	1170	18	0.9999	/	/	/

Table 2 Comparison between HPLC determination of wort carbohydrates with RI and ELSD detectors

N=16

	Fructose		Glucose		Sucrose		Maltose		Maltotriose	
	ELSD	RI	ELSD	RI	ELSD	RI	ELSD	RI	ELSD	RI
Mean	**2.0**	**2.1**	**15.6**	**13.8**	**3.1**	**2.3**	**85.5**	**64.9**	**20.0**	**14.8**
SD	0.6	0.8	1.7	2.8	0.9	0.6	7.8	7.6	2.1	2.6
RSD	30.1	38.7	10.8	20.3	27.9	26.2	9.1	11.8	10.5	17.8
RI/ELSD %	105		88		74		75		74	

3 Results and discussion

Preliminary analysis was carried out using the EBC [17] official method adapted using an amino column and acetonitrile/water as eluent.

The calibration data for the two detectors (ELSD and RI) are presented in Table 1. RI have a lower slope than ELSD. The fructose slope was about 10-fold lower, while for other sugars it was about 1/100th, meaning a low sensitivity of the RI detector.

On the other hand, both the poor resolutions obtained between maltose and maltotriose with the official method RI (Table 2), and the results obtained with this equipment, indicate that this detector is inadequate to completely estimate malt or beer carbohydrate composition. Sugars higher than DP6 are difficult to separate and to quantitate by RI; the peaks were too large and flattened because of the isocratic elution, and the run time was too long (Figure 1).

Only wort samples can be analysed and must be injected undiluted with a 20µl loop to adequately evaluate sugar, because of the low sensitivity of the RI detector utilised, under instrumental conditions (column, temperature and solvent) analogous to the ELSD detector.

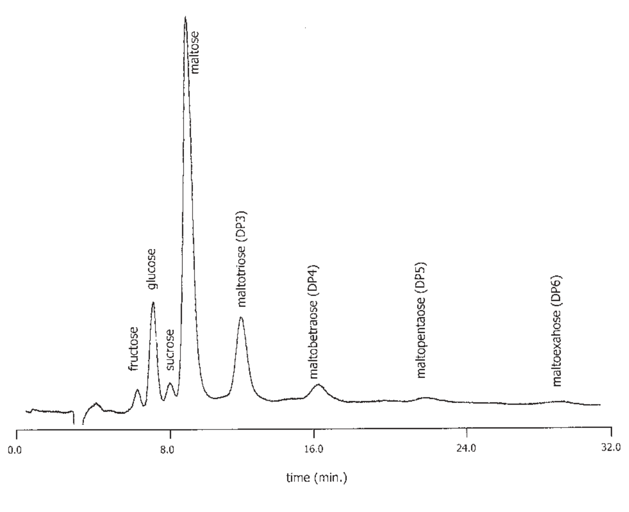

Fig. 1 HPLC-RI, sugar isocratic chromatogram of wort

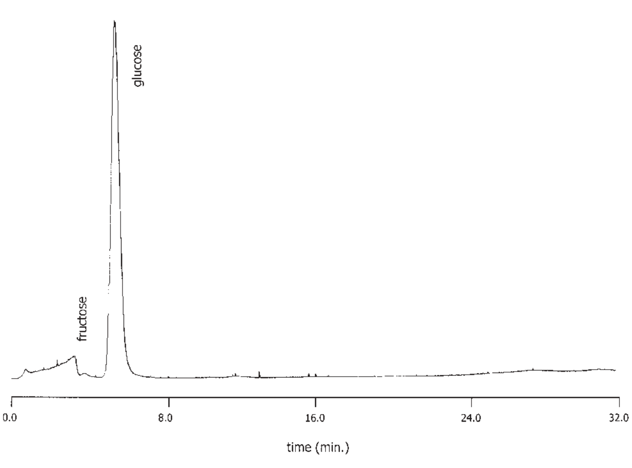

Fig. 2 HPLC-ELSD of a hydrolysed sample of wort

The calibration of the stock standard solution was performed in duplicate (Table 1). A correlation coefficient $R^2 \geq 0.99$ in a 0.3 ÷ 1.0 g/l range of concentration was obtained for oligomers up to maltotriose, and of 0.1 ÷ 0.5 g/l for oligomers DP4 up to DP7. The calibration showed different values for the area/concentration ratio, because the ELSD response can vary over time. It means that this kind of analysis requires the test of linearity and a daily screening of the stock standard solution.

The standard solution (diluted 1:2 for beer) simulated the amount of carbohydrates in wort and beer to minimize the error of determination.

Analyses of samples were initially performed in duplicate, but the high reproducibility of the method and daily checking of the detector conditions allowed a single chromatographic run to be made for each sample.

The low response in comparison to the sugar signals for beer, and the retention times, shorter than that for fructose, allowed organic acids not interfering in these analytics to be considered. The organic acids occurring in beer revealed with ELSD are mainly nonvolatile malic acid and citric acid [25]. A solution of malic and citric acids (250 mg/l each) was also analyzed under the same chromatographic conditions as used for sugar determination and were found to not interfere at all.

After acid hydrolysis of the beer and wort samples, the chromatographic determination of the hydrolyzed solutions showed peaks of glucose and fructose, only. The amount of both sugars increased in the hydrolyzed samples, and corresponded to the chemical determination reported as glucose units (Figure 2). For example, the glucose and fructose content of the hydrolyzed samples was 127.6 ± 3.4 gr/l by ELSD and 130.0 ± 2.3 gr/l by Fehling which were not found to be statistically different (N = 2, P = 0.01).

This confirms that the detected peaks are carbohydrates, and their calculated amount is reliable. The first eluting sugars (fructose, glucose, sucrose, maltose, and DP3 to DP7) were identified by comparing them to reference compounds. Other eluting sugars were identified as higher oligomers (DP8, DP9, etc.) in relation to their chromatographic behavior, their degradation products, and the amount compared with chemical analysis (Figure 3 and 4).

The peak resolution obtained under this procedure was excellent, and allowed to be separated and quantified 17 carbohydrates (Table 2). The results are in good agreement with those found in the literature. *Lehtonen* and *Hurme* [15] determined up to DP7 in beers, while *Uchida* et al. [22] found up to DP10.

Sugars over DP6 were regularly separated and detected, if occurring. In our beer samples for example, we easily quantified DP16.

The amount of the oligosaccharides DP8 to DP15 was indirectly estimated with respect to their glucose equivalent. The RRF (Relative Response Factor) was found to be rate constant from maltose up to DP7 (RSD <15%) as shown in Table 3, allowing to

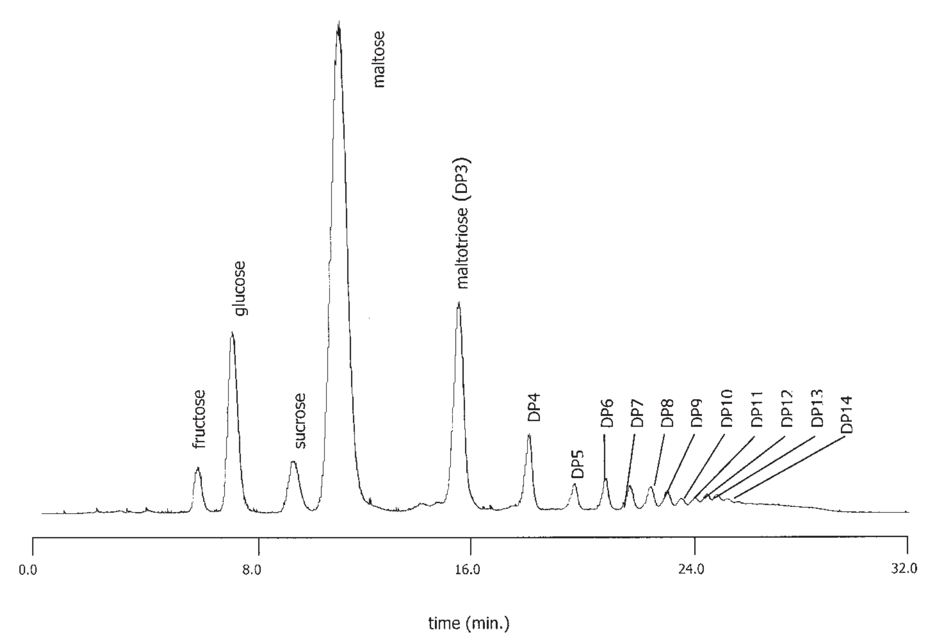

Fig. 3 HPLC-ELSD of a sample of wort

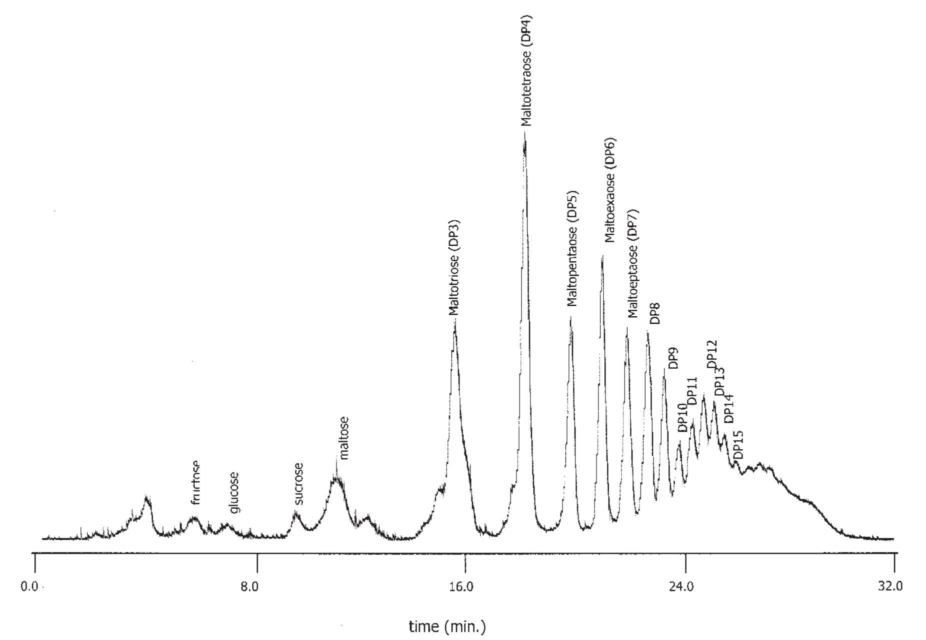

Fig. 4 HPLC-ELSD of a beer sample

Table 3 Relative response factors (RRF) and retention times of carbohydrates with ELSD detection

n.	Carbohydrate	RRF	RRT
1	**Fructose**	1.48	**1.0**
2	Glucose	1.10	1.2
3	Sucrose	1.88	1.7
4	**Maltose (DP2)**	**1.00**	2.0
5	Maltotriose (DP3)	1.02	2.6
6	Maltotetraose (DP4)	0.88	3.0
7	Maltopentaose (DP5)	0.79	3.2
8	Maltohexaose (DP6)	0.78	3.4
9	Maltoheptaose (DP7)	0.90	3.5
10	DP8	0.895*	3.6
11	DP9	0.895*	3.7
12	DP10	0.895*	3.8
13	DP11	0.895*	3.8
14	DP12	0.895*	3.9
15	DP13	0.895*	4.0
16	DP14	0.895*	4.1
17	DP15	0.895*	4.2

*Relative Response Factor (the area/concentration fraction with respect to the maltose) is the mean from maltose up to DP7.

utilize this mean ratio (0.895) to measure oligosaccharides higher than DP7. *Silbereisen* and *Bielig* [26] and *Otter* et al. [27] previously used similar procedures to evaluate maltose oligomers in the absence of reference compounds.

To compare the results of HPLC-ELSD determination to the chemical analysis of RS and TC, the oligosaccharide content was converted, calculating its glucose equivalent assuming that 2 glucose molecules condense and loose a water molecule. To calculate the TC content as glucose units, for each amount of oligosaccharide (g/l), the following formula was used:

$$TC = \frac{(g/l)}{MW} \times MW\,(glucose) \times DP$$

For RS calculation (except sucrose because it is non-reducing) the number of moles of each carbohydrate was multiplied for glucose molecular weight, because every oligosaccharide has the reducing power of glucose.

There is good agreement between HPLC-ELSD data and the chemical determinations as reported in Table 4. It confirms the correct assignment of the peaks and the quantitative evaluation of oligomers DP8 up to DP15.

In these analytical conditions, for beer samples, some minor peaks occurred near the main peaks of DP2 and DP3 (Figure 4). They were tentatively attributed to be sugar isomers (isomaltose, panose, isomaltosyl-maltose) according to their chromatographic behavior [28], because they disappeared after acid hydrolysis.

Table 4 Comparison between Chemical vs. HPLC-ELSD determination of malt syrup, wort and beer carbohydrates

	Total carbohydrates ELSD g/l	Total carbohydrates Fehling g/l	Reducing Sugar ELSD g/l	Reducing Sugar Fehling g/l
Mashed malt N=10				
Mean	**205.9** [a*]	**197.0** [a]	**97.6** [a]	**98.9** [a]
Mean Δ	20.6		11.4	
Mean Δ%	10.0		11.7	
Wort N=38				
Mean	**134.0** [a]	**134.4** [a]	**64.3** [a]	**66.7** [a]
Mean Δ	6.2		4.3	
Mean Δ%	4.6		6.6	
Beer N=24				
Mean	**31.6** [a]	**33.6** [b]	**7.6** [a]	**6.9** [a]
Mean Δ	2.6		1.1	
Mean Δ%	8.3		14.2	

* Means with different superscrits are significantly different at P ≤ 0.01
- "t" test, coupled sample
- "1-t"-test

Table 5 Carbohydrates in mashed malt, wort and beer (mg/l)						
Carbohydrate	**Mashed malt**		**Wort**		**Beer**	
	mean	sd	mean	sd	Mean	sd
Fructose	3.7	0.6	2.1	0.3	0.4	0.1
Glucose	19.4	2.7	13.8	2.1	0.1	0.0
Sucrose	5.8	1.3	3.0	0.7	0.6	0.2
Maltose (DP2)	114.8	13.9	73.5	5.4	3.8	3.4
Maltotriose (DP3)	27.2	2.7	19.6	2.2	4.3	0.8
Maltotetraose (DP4)	7.8	2.7	5.0	1.2	5.5	0.6
Maltopentaose (DP5)	2.7	1.2	1.6	0.2	2.2	0.3
Maltohexaose (DP6)	3.1	1.2	1.8	0.3	2.7	0.3
Maltoheptaose (DP7)	2.6	0.6	1.5	0.2	2.2	0.3
DP8	2.2	0.5	1.3	0.1	2.2	0.4
DP9	1.5	0.6	1.1	0.3	1.8	0.4
DP10	0.6	0.3	0.4	0.1	0.5	0.1
DP11	0.5	0.1	0.2	0.0	0.4	0.0
DP12	0.6	0.1	0.3	0.0	0.4	0.1
DP13	0.4	0.1	0.3	0.1	0.3	0.1
DP14	0.2	0.1	0.1	0.1	0.1	0.1
DP15	0.1	0.0	0.0	0.0	0.1	0.0

n=10 for mashed malt; n=38 for wort; n=24 for beer

The comparison between HPLC-ELSD and chemical analysis gives useful information about RS and TC. Furthermore, chemical analysis is a definite reference parameter for HPLC analysis. On the contrary, the comparison between HPLC-RI and chemical analysis is not possible because not all the oligomers are analysed with HPLC-RI.

Mashed malt had a composition very similar to the wort, because it was collected at the end of the mashing process, just before filtration, but generally the results of the former are not in agreement with the chemical evaluation because its sugars rapidly degrade under enzyme and microbiological activity. A carbohydrate degradation of 30% occurred after 3 – 4 hours after thawing.

The amount of beer carbohydrates evaluated by chemical analysis were slightly higher than those evaluated by HPLC-ELSD (Table 4). This is probably due to the presence of fermentation products reducing Fehling reagent as well.

The HPLC-ELSD and carbohydrate chemical analysis of mashed malt and wort gave similar results (Table 4). Considering that in these samples the carbohydrate content is high and there is an absence of interfering compounds the good agreement between the sugar content determined by HPLC-ELSD and the respective estimated amount determined by chemical analyses confirm the reliability of the proposed procedure.

Mashed malt and wort contain mainly low molecular weight and fermentable carbohydrates (Table 5), making up to 88% of the total amount. Oligomeric and non fermentable carbohydrates also occur in a lower amount (12%).

The opposite occurs in beer, in which, after fermentation, the main carbohydrates are the non-fermentable oligomers (71%). Fermentable sugars occur only in a minor amount.

The amount of carbohydrate found in Italian lager worts and beers is in good agreement with those found in the literature [5] [6] and those published in previous papers for Italian beers [4].

4 Conclusions

HPLC analysis of carbohydrates in brewing production, enables the trend for better process control and evaluation of beer quality.

With HPLC coupled with ELSD it is possible to separate and quantify individual carbohydrates, either fermentable (fructose, glucose, sucrose, maltose and maltotriose) or non-fermentable (maltotetraose to maltose polymer containing up to 15 glucose units) in less than 40 minutes.

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5 Zusammenfassung

Floridi, S., Miniati, E., Montanari, L., und Fantozzi, P.: Bestimmung von Kohlenhydraten in Würze und Bier mit HPLC-ELSD — Monatsschrift für Brauwissenschaft 54, Nr. 9/10, 209 – 215, 2001

BC 35 Würze

Eine vollständige Trennung vergärbarer und nicht vergärbarer Kohlehydrate von bis zu 15 Glucoseeinheiten wurde in Würze, Maische und Bier in 40 Minuten durch direkte Injektion in einer NH_2-Trennsäule und einen HPLC-ESLD-Apparat erzielt. Die Ergebnisse waren vergleichbar mit denen der offiziellen HPLC-RI-Methode und der chemischen Bestimmung mit dem Fehling-Reagenz. In italienischer Würze und Maische waren ungefähr 88% der gesamten Kohlenhydrate vergärbar, in Bier dagegen nur 29%.

Floridi, S., Miniati, E., Montanari, L., et Fantozzi, P.: Détermination des glucides dans le moût de bière et dans la bière par HPLC-ELSD
— Monatsschrift für Brauwissenschaft 54, Nr. 9/10, 209 – 215, 2001

BC 35 Moût

La séparation totalen de glucides fermentescibles et non fermentescibles jusqu'à 15 unités de glucose dans le moût de bière, dans la maische de malt et dans la bière a été obtenue en 40 minutes par injection directe dans une colonne NH_2 et un appareil HPLC-ESLD. Les résultats sont comparables à ceux obtenus par des procédés officiels de HPLC-RI et par la détermination chimique utilisant le réactif de Fehling. Dans des moûts italiens et dans la maische de malt, environ 88 % des glucides totaux sont fermentescibles et dans la bière seulement 29 % sont fermentescibles.

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