

# Determination of Carboxylic Acids, Carbohydrates, Glycerol, Ethanol, and 5-HMF in Beer by High-Performance Liquid Chromatography and UV–Refractive Index Double Detection

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## Abstract

A high-performance liquid chromatographic method is proposed for the simultaneous separation of main carboxylic acids, carbohydrates, ethanol, glycerol, and 5-HMF in beer by direct injection. A column packed with a sulfonated divinyl benzene–styrene copolymer and an isocratic elution with 0.0045N sulfuric acid and acetonitrile (6%, v/v) are employed. UV and refractive index detectors connected in series are also used to reduce the matrix interference of phenolic compounds. In conditions described, nine compounds are quantitated in a single chromatographic run without any pretreatment except for sample dilution and filtration before injection. Precision, accuracy, linearity of response, limit of detection, and limit of quantitation are also evaluated for each compound. Satisfactory results are obtained to justify the application of this method to all phases of beer production for process and quality control.

## Introduction

The determination of carbohydrates, organic acids, glycerol, and ethanol is usually required for process control and the evaluation of quality in many food and beverage industries (1–3). Furanic compounds such as 5-hydroxymethylfurfural (5-HMF) are well-known markers for heat treatment in various food and beverages containing proteins and carbohydrates. For their nutritional, sensorial, and technological importance, a number of high-performance liquid chromatographic (HPLC) methods have been proposed to determine these compounds of different natures (i.e., organic acids, sugars, alcohol, and 5-HMF) in different food and beverages (4–8).

In the brewing industry, specific chromatographic methods have been developed in order to evaluate the single classes of

these compounds (9–17). However, it would be interesting to quantitate the main compounds of each class in a single-run separation, but when the analysis was carried out by directly injecting the sample using columns packed with a sulfonated divinyl benzene–styrene copolymer (DVB-S), a poor resolution of some of the compounds was often observed (6–8,18–21).

In order to avoid these interferences, some authors (6,21) proposed either a dual-column system or UV and refractive index (RI) detectors connected in series. The use of the RI detector seemed to reduce interference from phenolic compounds when organic acids were evaluated in grape musts and wines (2,20).

In order to optimize the separation, a sample cleanup was proposed prior to HPLC analysis using both solid-phase extraction (SPE) cartridges and ion-exchange resins (7,19–21). Sample cleanup procedures however increase the costs and the analysis time, making both process control and routine analyses time consuming and expensive.

The aim of this work was to develop a simple HPLC separation method (direct-injection, single-run analysis) to quantitate some of the most important compounds influencing beer quality. The chromatographic conditions were chosen to limit the matrix interference and improve separation by reducing the retention time of furanic compounds. For this reason both a double detection (UV-RI) and a mobile phase containing acetonitrile as an organic modifier were used. Accuracy, precision, and linearity of the method were determined on two different types of commercial beer. Finally, the possibility to evaluate the total dry extract using the same HPLC method was also considered.

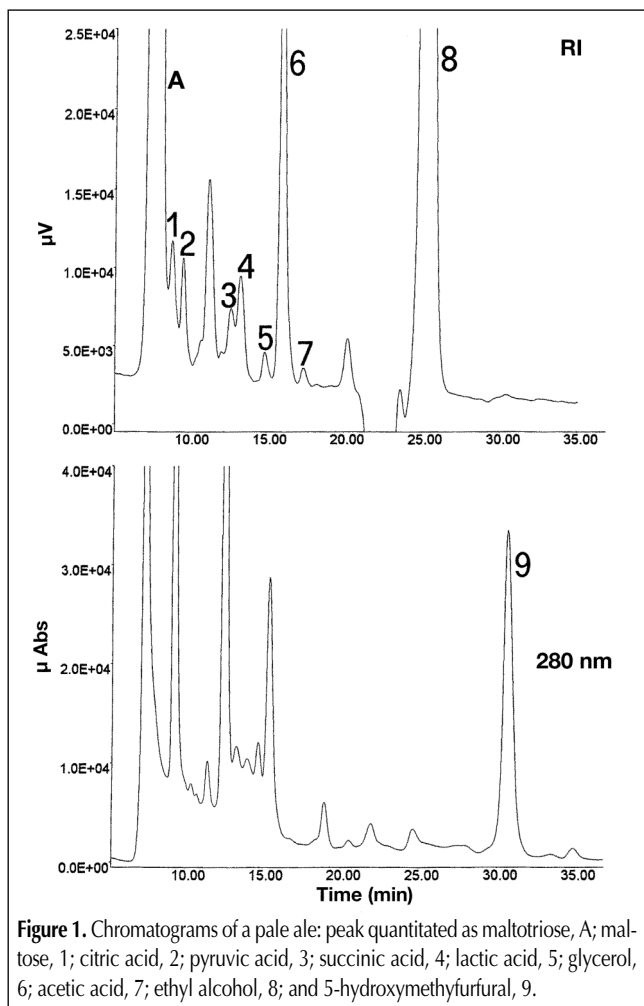
## Experimental

### Materials

Samples of pale and mild ale were purchased in retail stores and stored at 4°C until analysis. HPLC-grade acetonitrile, HPLC-grade water, and sulfuric acid were supplied by Carlo Erba (Milan,

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Italy). A standard solution of 5-HMF, glycerol, ethanol, D(-)fructose, D(+)-glucose, maltose, acetic, citric, D,L-lactic, pyruvic, and succinic acid (Sigma-Aldrich, Milan, Italy) were prepared by dissolving known amounts of analytical-reagent-grade chemicals in HPLC-grade water.



**Figure 1.** Chromatograms of a pale ale: peak quantitated as maltotriose, A; maltose, 1; citric acid, 2; pyruvic acid, 3; succinic acid, 4; lactic acid, 5; glycerol, 6; acetic acid, 7; ethyl alcohol, 8; and 5-hydroxymethylfurfural, 9.

### HPLC analysis

Analysis was carried out using a PU 980 pump equipped with UV 970 and RI 830 detectors (Jasco International Co., Tokyo, Japan) connected in series.

Chromatographic separations were performed on an Aminex HPX-87H column (300 × 7.8 mm) (Bio-Rad Laboratories, Richmond, CA) with a precolumn (30 × 4.6 mm) of the same stationary phase (DVB-S, hydrogen form). The column, precolumn, and the 7515 injection valve (Rheodyne, Cotati, CA) were kept at 45°C using a heating block (Jones Chromatography, Hengoed, U.K.).

Isocratic elution at a flow rate of 0.5 mL/min was carried out using a mixture of 0.0045N sulfuric acid and acetonitrile (6%, v/v). Peak detection was made using the UV detector set at 280 nm, and the cells of the RI detector were kept at 40°C. The samples were appropriately degassed, twice diluted with double-distilled water, filtered through a Cameo 0.22- $\mu$ m filter membrane (MSI, Westborough, MA), and then injected (20- $\mu$ L loop volume). Data were obtained and processed using 1.0 BORWIN chromatography software (JMBS Developpements, Grenoble, France).

Peak identification was carried out by spiking the beer sample with pure standards and comparing the retention times with those of pure compounds.

### Total dry extract

The extract of 22 beers was evaluated using the Association of Official Analytical Chemist (AOAC) method (22). Following this procedure, the beers were evaporated down to one-third of their original weight, and then the initial sample volume was reconstituted with water. Values of the totally dry extract were obtained from the density of the reconstituted sample density measured using a hydrostatic balance (Densi-Mat Gibertini, Milan, Italy), then the obtained density values were converted to totally dry extract by using the published table (22).

## Results and Discussion

Figure 1 shows two chromatograms of a pale ale. Under our

**Table I.** Linearity, LOD, and LOQ for the Standard Calibration Curves

	Retention time (min)	Calibration curve*					LOD (mg/L)	LOQ (mg/L)
		Concentration range (g/L)	Correlation coefficient ( $n = 7$ )	Slope	Intercept			
Peak A <sup>†</sup>	7.50	0.32–10.12	0.999	917,266	-29,690	n.d. <sup>‡</sup>	n.d.	
Maltose	8.95	0.18–12.55	0.998	920,371	25,834	1.5	4.5	
Citric acid	9.63	0.03–0.52	0.999	2,343,300	2161	1.3	3.9	
Pyruvic acid	12.68	0.03–0.53	0.999	9,803,004	33,847	1.7	5.1	
Succinic acid	13.22	0.04–1.04	0.997	3,032,159	-26,440	1.5	4.5	
Lactic acid	14.88	0.03–0.95	0.999	2,155,875	-3953	1.5	4.5	
Glycerol	16.00	0.01–2.03	0.999	832,646	2349	2.6	7.8	
Acetic acid	17.37	0.04–0.58	0.998	1,164,382	876	3.0	9.0	
Ethanol	25.25	6.14–98.3	0.999	318,492	124,784	8.1	24.3	
5-HMF <sup>§</sup>	30.56	1–128	0.999	281,626,290	417,139	0.012	0.036	

\* Peak area = (standard concentration × slope) + intercept.

<sup>†</sup> Quantitated as a maltotriose.

<sup>‡</sup> n.d., not determined.

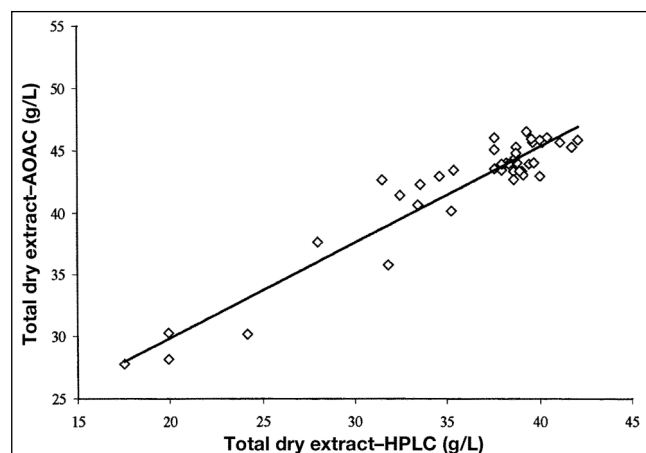
<sup>§</sup> Concentrations of 5-HMF are expressed in milligrams per liter.

conditions the main organic acids were separated from carbohydrates and other compounds within 30 min (Table I). A comparison of RI and UV traces indicated that interfering compounds (probably phenols with relevant absorbance at 280 nm), which would make the estimation of the citric, pyruvic, and lactic acid levels difficult (19), were not detected by the RI system.

No detectable amounts of glucose and fructose were found in the commercial beer sample; therefore, these compounds were not considered further in our work. Moreover, the injection of standard solutions of glucose and fructose indicated no coelution with other compounds (their retention times were 10.85 and 11.82 min, respectively).

The relationship between the peak area and the concentration was considered to be linear for the whole examined concentration range (Table I). In accordance with the signal-to-baseline noise ratio (S/N), the lowest instrumental limits of detection (LOD) and quantitation (LOQ) for single compounds were calculated as the concentration of a standard solution that produced a peak height corresponding to S/N = 3 and S/N = 9, respectively.

Under our conditions the LOQ for organic acids ranged from 4



**Figure 2.** Correlation between the dry extract values obtained using the official AOAC method and those obtained using the HPLC method calculated as the sum of peak A, maltose, organic acids, 5-HMF, and glycerol. AOAC result =  $(0.76 \times \text{HPLC result}) + 14.95$ ,  $R^2 = 0.89$ ,  $p < 0.01$ ,  $n = 22$ .

to 9 mg/L (Table I). These values appeared to be significantly higher than those reported in other studies (9,23–25), but the RI detector was considerably less sensitive than UV detectors. However, it should be emphasized that beer generally contains these compounds in higher quantities, thus the observed LOQ values may be considered satisfactory for practical application in beer production and quality-control processes. Accuracy was evaluated by adding known amounts of standard compounds to two different samples of the commercial beers (Table II).

Expected concentrations were calculated as the sum of the original amounts in the sample and the added amounts. The original amounts were estimated analyzing the samples in triplicate with the proposed HPLC method. Recoveries were calculated for each compound as the percent ratio between the observed and expected values. The average recovery ranged from 102% to 93% with a relative standard deviation always better than 7%.

Repeatability of the chromatographic analysis was evaluated for the same beer sample analyzed by the same operator five times a day for three consecutive days.

Intraday and interday repeatability values were estimated both for the retention time and the peak area. The relative standard deviations for the peak area were under 5%, and the repeatability values for the retention time were under 0.2% (Table III).

A large, wide peak was observed on the RI detector at the beginning of the chromatographic separation (peak A in Figure 1). According to the technical notes of Bio-Rad Laboratories, the Aminex separation column also works on the physical exclusion of the molecules that are too large to deeply penetrate the pore structure of the resin. Because of this, it can be assumed that peak A may be made up of unretained polysaccharides, starch, and dextrin, which are detectable with RI. The area of this peak was thus quantitated as a maltotriose equivalent (g/L), and the value obtained was added to those of maltose, organic acids, 5-HMF, and glycerol to give an estimated HPLC value of the dry sample extract.

Figure 2 shows the significant correlation between the AOAC method and the HPLC results even though the HPLC method usually gives underestimated values when compared with the official method. The HPLC method doesn't give a correct estima-

**Table II. Accuracy Determined Using the Standard Addition Method**

	Pale ale				%Average recovery (and RSD*)	Mild ale				
	Original amount (g/L)	Added amount (g/L)				Original amount (g/L)	Added amount (g/L)			
Maltose	0.37	0.30	0.60	0.90	99.3 (0.5)	1.25	1.25	2.50	3.75	97.8 (1.03)
Citric acid	0.08	0.05	0.10	0.15	99.7 (1.2)	0.19	0.18	0.36	0.54	100.7 (2.36)
Pyruvic acid	0.08	0.09	0.18	0.27	99.2 (0.5)	0.21	0.31	0.62	0.93	99.1 (0.56)
Succinic acid	0.05	0.02	0.04	0.06	98.0 (5.72)	0.16	0.15	0.30	0.45	99.8 (1.62)
Lactic acid	0.04	0.01	0.02	0.03	101.7 (2.36)	0.08	0.09	0.02	0.27	99.6 (1.00)
Glycerol	0.43	0.41	0.82	1.23	97.7 (1.25)	1.30	1.20	2.40	3.60	100.2 (1.36)
Acetic acid	0.06	0.01	0.02	0.03	93.3 (7.07)	0.05	0.01	0.02	0.03	96.3 (1.25)
Ethanol	35.2	9.8	19.6	29.4	99.2 (0.62)	33.9	10.0	20.0	30.0	97.3 (2.62)
5-HMF†	1.21	1.02	2.04	3.06	97.3 (1.25)	3.88	3.10	6.20	9.30	99.5 (1.39)

\* RSD, relative standard deviation.

† Concentrations of 5-HMF are expressed in milligrams per liter.

**Table III. Repeatability Trials (Interday and Intraday) for Retention Times and Peak Areas\***

	Retention time		Peak area	
	RSD <sup>†</sup> intraday	RSD interday	RSD intraday	RSD interday
Peak A	0.11	0.13	0.50	0.65
Maltose	0.08	0.10	0.60	0.75
Citric acid	0.05	0.10	1.40	2.45
Pyruvic acid	0.06	0.08	1.75	2.40
Succinic acid	0.13	0.16	1.50	1.90
Lactic acid	0.09	0.12	2.10	2.35
Glycerol	0.04	0.07	1.10	1.70
Acetic acid	0.14	0.14	3.25	4.05
Ethanol	0.02	0.09	0.80	0.90
5-HMF	0.05	0.09	1.70	1.90

\* Values are the average of the data for pale and mild ales.  
<sup>†</sup> RSD, relative standard deviation.

tion of the beer extract, but it may be useful to compare very similar products or continuously follow the evolution and the fermentation rate of beer during the production process.

## Conclusion

The proposed HPLC method allowed for the evaluation of nine different beer compounds in a single chromatographic run with a total analysis time of 35–40 min. The use of a column packed with DVB-S reduced treatments of the sample that was diluted and directly injected. Simple chromatographic conditions increased the accuracy, repeatability, and speed of analysis. The LOQ was satisfactory in order to justify the application of this method for production and quality assurance of which there is a need for reliable, routine controls in order to follow the status of beer formation.

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