HPLC Analysis

The Role of Ultrapure Water

HPLC is an analytical procedure for separation, identification and quantification of substances using liquid chromatography. The beginnings of HPLC – High Pressure Liquid Chromatography – go back to the 60's. Thanks to improved column materials and equipment, it has come to be known as High Performance Liquid Chromatography since the end of the 70's [1].

Introduction

In HPLC, the mixture to be separated is transferred to a column with a solvent (eluting agent) or with a solvent mixture (eluant/mobile phase), by an injector and a pump. The column is a tube, in most cases of stainless steel, filled with the so-called stationary phase (see also Fig. 1). The stationary phase usually consists of porous silica gel or polymer particles with chemical ligands bound on their surface. These ligands are responsible for the selective interactions between the analytes and the stationary phase, which are necessary for effective chromatographic separation. Depending on the sample and stationary phase, the separation mechanisms involved are, for example, adsorption by Van der Waals forces, ion exchange, ion exclusion, etc. The substances of a sample are retained on the column packing material for different lengths of time and therefore exit the column after different retention times. The individual components of the sample are then registered by a detector and evaluated by a computer. The result is a chromatogram (Fig. 1, 2, 3). The number of peaks corresponds to the number of separated components in the sample, and the area is proportional to the concentration of these separated components [1].

Among the typical applications for HPLC is sugar analysis. This was performed within the scope of various tests conducted in order to characterize the quality of membranes. On the one hand, membranes were tested for their ability to remove sugar molecules and, on the other hand, the activity of enzyme-immobilized membranes was determined. For this purpose, sugars, such as raffinose, glucose and fructose, were assayed. These types of sugar can be specifically detected by using enzymatic methods like the GOD/POD assay for glucose [2] or spectroscopic methods, such as determination of fructose [3].

In advanced analysis, sugar is now frequently assayed by thin-layer chromatography (TLC), gas chromatography (GC) and high-performance liquid chromatography (HPLC). These methods are used especially when mixtures containing several types of sugar must be separated [4].

In HPLC as described here, the eluant must have especially high physical and chemical purity, and may not contain suspended mechanical particles or any dissolved substances that can be released by the column at a delayed time and thus generate a signal. The quality of a solvent is often decisive for the reliability of an HPLC analytical run. The presence of trace contaminants during gradient elution can result in "ghost or phantom peaks". Such trace substances accumulate in the column during an analytical run and are increasingly released when the eluting agent is subsequently changed. Water used as an eluant must be free of microorganisms. For this purpose, substances that prevent the growth of microbes and algae in the solvent mixture, such as copper salts or sodium azide, can be added [5]. In doing so, the column manufacturer's recommendations need to be followed as the use of incorrect additives can result in irreversible damage to the column.

Deionized or distilled water still contains considerable quantities of organic substances, which can cause ghost peaks [5]. Contaminated solvents can lead to the buildup of deposits on the stationary phase and thus result in blockage of the column, which would be manifested by an increase in pressure and a shift in the running time for the samples.

Purified Water for Use as an Eluant

Water of the special quality required for HPLC can be purchased from various manufacturers or be produced directly on site for use on demand by employing a water purification system. The following describes the tests performed for separation of sugar mixtures in which ultrapure water was used as the mobile phase (eluant).

Materials and Method

The samples were analyzed using an HPLC 1200 Series (Agilent) system with a Rezex RNM Carbohydrate Na^+ 8% HPLC column (Phenomenex)[6]. This column is filled with a cross-linked polystyrene-divinylbenzene-copolymer modified by sodium sulfonate groups and uses an ion exclusion mechanism. This means that analytes are separated based on different ionic interactions. Because of the sulfonate groups on the surface of this column packing material, the pores have a negative charge. As a result, negatively charged molecules cannot penetrate into the pores of the material, which causes them to elute earlier. This ion exclusion mechanism is based on Gibbs-Donnan equilibrium that governs the behavior of ions near a membrane. Analytes that are able to penetrate into the membrane's pores are subsequently separated based on steric differences as well as on hydrophobic and polar interactions with the functional groups on the surface of the stationary phase. For more details on this separation mechanism, please refer to [7].

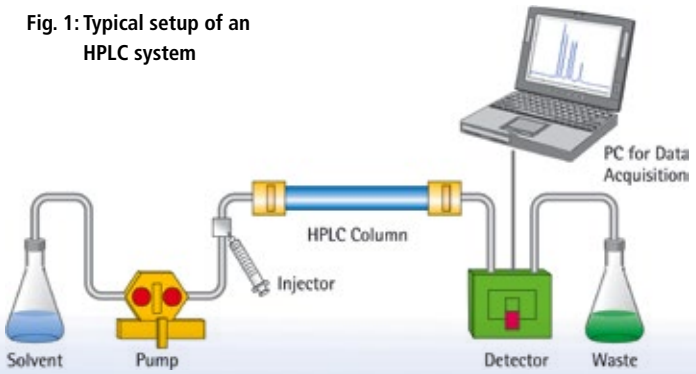

Fig. 1: Typical setup of an HPLC system

Flow rate	[ml/min]	0.6
Time	[min]	25
Maximum pressure	[bar]	70
Temperature of the Column Compartment	[°C]	75
Temperature of the RI detector	[°C]	35
Injection volume	[µl]	2

Table 1: HPLC method

The retention times for various types of sugar are determined by the absorbance of the refractive index (RI) signal. This RI signal is expressed as a dimensionless number in nRIU (nano Refractive Index Units) and indicates the difference between the refractive index of the sample in the sample cell and the mobile phase in the reference cell.

Ultrapure water produced by the Arium pro VF (Sartorius) system was used as the mobile phase (a detailed description of this system see page XXX). For degassing the eluant in the HPLC system, this ultrapure water was filtered by vacuum through a Bottle Top disposable unit equipped with a 0.2 µm membrane.

Procedure for HPLC Analysis

To prepare for the analytical runs, the column was heated to 75°C in the column compartment (heater) and flushed overnight with ultrapure water at 0.6 ml/min. The optical unit of the RI detector was heated to 35°C. Samples to be analyzed were prepared using ultrapure water and prefiltered through a 0.2 µm syringe filter unit. The samples were analyzed using HPLC according to the parameters defined by an HPLC method [6] (Table 1).

Results

To determine the retention times of the individual types of sugar (Table 2), these were prepared and injected individually (Fig. 2). As different sugars interact with the stationary phase to a varying degree, specific retention times are recorded by the RI detector once each sugar has moved through the column.

Concentration [mg/ml]	Retention Time [min]	Peak Area [nRIU*s]
0	-	-
0.015	8.96	603
0.03	8.96	1,088
0.06	8.96	2,327
0.125	8.96	4,178
0.25	8.96	7,607
0.5	8.96	15,097
1	8.96	30,495

Table 2: Reproducibility of the Retention Times and Determination of the Detection Limit using a Raffinose Standard Series as an Example

After the individual types of sugar had been determined, a sugar mixture was prepared and separated (Fig. 2).

The individual sugar components were separated from one another. The peaks for the different retention times could be allocated to the individual sugar samples assayed.

The effect of contaminants, or the influence of salts, was simulated by injecting potassium phosphate buffer and tap water (Fig. 3).

Injection of tap water with a conductivity of 265 µS/cm and of potassium phosphate buffer with a conductivity of 1,700 µS/cm showed clear signals and can therefore be distinctly identified as contamination.

Multiply-charged ions are especially prone to binding with sulfonate groups. This alters the dissociation equilibrium and can affect the retention time for a particular

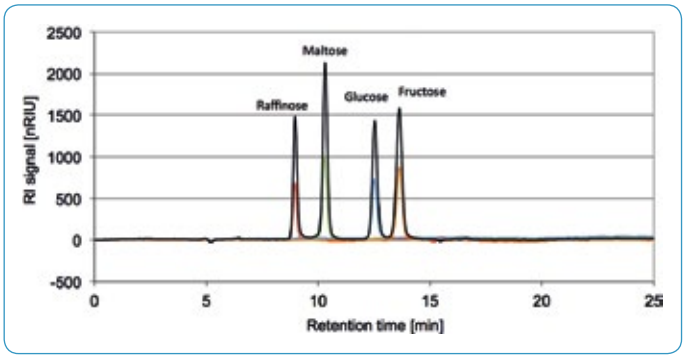

Fig. 2: Separation of individually injected sugar samples and of a sugar mixture using a RNM Carbohydrate Na⁺ 8% column with ultrapure water. Black curve: sugar mixture

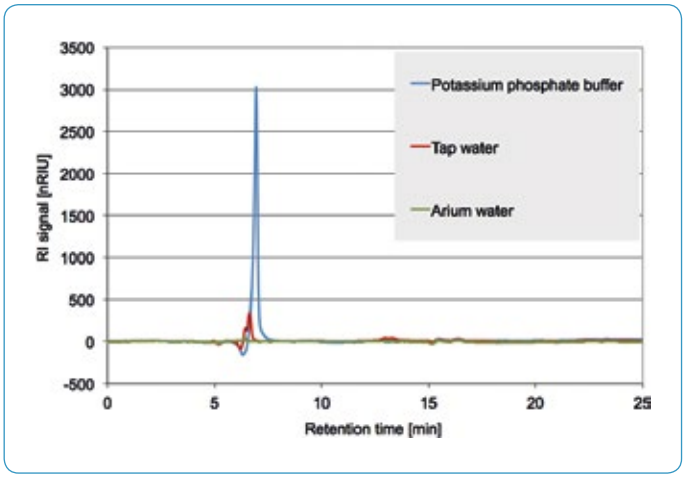

Fig 3: Chromatograms of 2 µl of 10 mM of potassium phosphate buffer, 2 µl of tap water and 2 µl of Arium ultrapure water

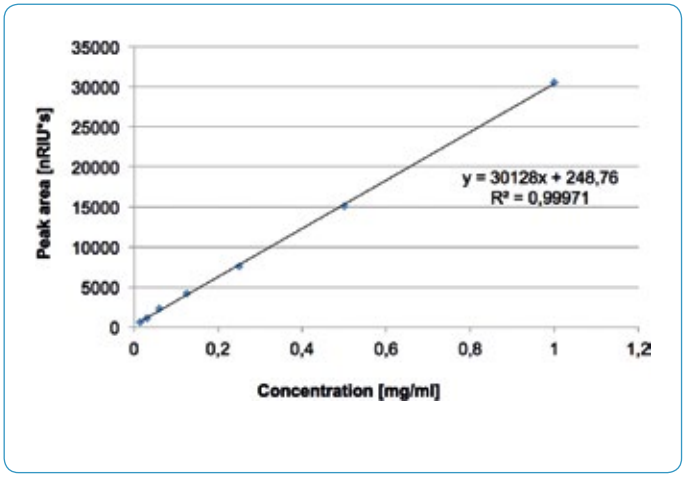

Fig. 4: Standard series for raffinose (values from Table 2) passed through a Rezex RNM Carbohydrate Na⁺ 8% column with Arium pro VF ultrapure water

sugar. For this reason, the mobile phase must be free of salts and other contaminants in order to perform reliable HPLC analysis with stable retention times and to avoid ghost peaks. The ultrapure water used in this analysis has a conductivity of 0.055 µS/cm and is largely free of interfering contaminants, which is expressed as a flat baseline without peaks (see green baseline in Fig. 3).

The column pressure during the analytical runs consistently remained at 23 bar (~345 psi). This shows that no deposits had built up in the column. Blank runs at the beginning and the end did not show any change; i.e., there were no contaminants in the mobile phase.

Standard series with different concentrations were analyzed to determine the reproducibility and detection limit. Raffinose is given as an example of these series performed. The retention times and peak areas were recorded and are listed in the Table 2.

The consistent retention times repeatedly obtained show excellent reproducibility. The raffinose standard series shows a linear curve up to a concentration of 0.015 mg/ml (Fig. 4).

By generating a standard strait line based on the peak areas a quantification of the sample, in this case raffinose, is possible.

Conclusion

The results show that ultrapure water can be readily used as the mobile phase for HPLC analysis of the water-soluble saccharides described in this paper. The interactions of the sample with the stationary phase are not affected by the mobile phase as the ultrapure water that is produced with a conductivity of 0.055 µS/cm can be considered virtually free of contaminants. As a result, there are no salts present that would otherwise cause ghost or phantom peaks [5]. In addition, the results of the trials lead to the conclusion that no deposits occur on the stationary phase, which would otherwise be manifested by an increase in pressure and a shift in the running times of the samples.

Therefore ultrapure water is an affordable alternative to commercially sold ultrapure water to prepare high-purity eluants for HPLC analysis, as used in food analysis, environmental analysis, as well as in medical, chemical and biochemical research and during in-process quality control testing in the pharmaceutical and biotech industries. The studies performed ultrapure water as a mobile phase in HPLC are to be extended in the near future to other separation technologies, such as reversed phase chromatography, size exclusion chromatography or ultrahigh-performance liquid chromatography.

References

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