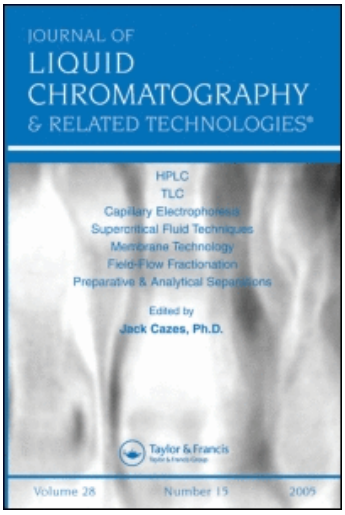

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Column Liquid Chromatography of Reducing Carbohydrates by Fluorometric Reaction Detection with a Pressurized Reactor Outlet

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To cite this Article Berg, J. H. M. van den , Horsels, H. W. M. and Deelder, R. S.(1984) 'Column Liquid Chromatography of Reducing Carbohydrates by Fluorometric Reaction Detection with a Pressurized Reactor Outlet', Journal of Liquid Chromatography & Related Technologies, 7: 12, 2351 — 2365
To link to this Article: DOI: 10.1080/01483918408068882
URL: http://dx.doi.org/10.1080/01483918408068882

JOURNAL OF LIQUID CHROMATOGRAPHY, 7(12), 2351-2365 (1984)

COLUMN LIQUID CHROMATOGRAPHY OF REDUCING CARBOHYDRATES BY FLUOROMETRIC REACTION DETECTION WITH A PRESSURIZED REACTOR OUTLET*

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ABSTRACT

The use of reaction detection in column liquid chromatography is not always precluded by the limitation of the reaction time to one or two minutes. A closed pressurized reactor allows heating of the reaction mixture to temperatures that exceed the boiling point of the mixture to enhance reaction speed.

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* Part of this paper has been presented at the Seventh International Symposium on Column Liquid Chromatography, Baden-Baden, G.F.R., 1983.

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 0148-3919/84/0712-2351$3.50/0

INTRODUCTION

The translation of classical analytical procedures in which chromophoric or fluorophoric products are formed to flow systems is often encountered in modern liquid chromatography and flow injection analysis literature. Reaction detection considerably extends the application potential of column liquid chromatography (1). In reviews and comparative studies (1-4) on the subject of the performance of post-column reactors in column liquid chromatography, the residence time of the components in the reactor determines the applicability of different types of reactors. For fast reactions (residence time 1 minute), most of the reactors investigated meet the requirements set by state-of-the-art column liquid chromatography. However, for reaction times exceeding 5 minutes the performance of all reactor types falls short of the demands of modern LC, with the exception of gas-segmented flow reactors without a debubbler, which are not commercially available yet. However, the packed reactor and the tubular reactor have distinct advantages. The latter is commercially available and widely used as post-column reactor. It should be emphasized that the limitation of the reaction time to one or two minutes precludes the use of inefficient reaction detectors. Slow reactions can often be accelerated considerably by heating. When tubular or packed reactors are used a reactor with a pressurized outlet allows the reaction mixture be heated to above its boiling point.

This is demonstrated in the present study for the determination of reducing carbohydrates by the reaction with

2-cyanoacetamide. 2-Cyanocetamide reacts with carbohydrates in a slightly alkaline medium to yield intensely fluorescent products (5).

EXPERIMENTAL

Equipment

The chromatographic system, including separation column, tubular reactor and cooler, is shown in Figure 1. High pressure pumps from various manufacturers (type HP1081B, Hewlett Packard, Waldbronn, G.F.R.; type SP 740B, Spectra Physics, Santa Clara, CA, U.S.A.; type M6000A, Waters Associates, Milford, MA, U.S.A.) were used as solvent and reagent delivery systems. Sampling valves (type 7010 and 7410, Rheodyne, Berkeley, CA, U.S.A.) with 5 and 20 µl loops and a 1 µl internal loop respectively, were used for injecting inert tracers or reactants. A filter fluorometer (type 420C/E, Waters Associates) equipped with a low pressure mercury lamp (type F4T5/BL) covered with a smooth layer of crystalline phosphorus and further equipped with a bandpass filter at 337 mnm as excitation filter and a cut-off filter at 375 nm as emission filter was employed as detector. Later, for comparison a refractive index detector (type 79877A, Hewlett Packard) was used instead of the fluorometric reaction detector.

Capillary tubes (316 SS) of 20 m and 15 m (ID 0.25 mm) and 2 m (ID 0.10 mm) all with an OD of 1/16" were purchased from Handy & Harman (Norristown, PA, U.S.A.) and wound to coils with a diameter

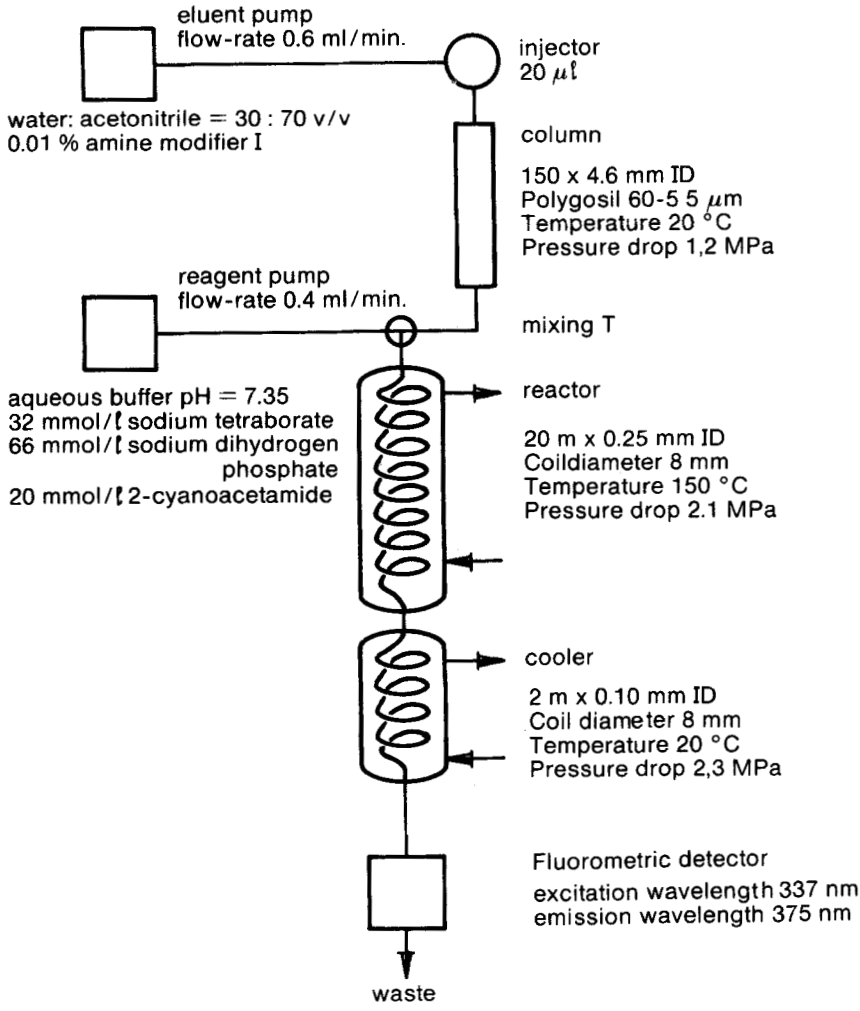

FIGURE 1

Schematic diagram of the liquid chromatographic equipment with post-column detection system.

of 8 mm. The tubular reactor was held at constant temperature by silicone oil, which was circulated by a thermostat (type U3 S15/12, Lauda, Königshofen, G.R.F.) with an accuracy of 0.5 °C. The reactor was mounted in a SS vessel. The cooler is also held at constant temperature (20 °C) by means of a thermostat of the same type. The estimated back pressure required to keep the effluent from the reactor in the liquid phase is 0.6 MPa. For safety reasons the cooler was designed to produce a back pressure of 2.3 MPa. A 150 mm long 316 SS liquid chromatographic column (type Lichroma, Handy & Harman) with an ID of 4.6 mm was filled with silica (type Polygosil 60-5, mean particle size 5 μm, Macherey & Nagel, Düren, G.F.R.) by means of a slurry technique. The column was thermostatted by means of an oven at 20 °C in the HP 1081 B liquid chromatograph.

The detector signal was digitized by means of a digital voltmeter (type HP3473A, Hewlett Packard) and the peaks were reconstructed by batchwise processing of the data on a desk computer (type HP 85, Hewlett Packard). The same procedure for data collection was used, when calculation of second moments and deconvolution according to Yau et al (6) were required. Peak area measurements and data handling were done with a chromatographic datasystem type SP 4000, Spectra Physics).

Chemicals

All reagents and pure analytical reference chemicals were of pro analyse grade and had been purchased from Merck (Darmstadt,

G.F.R.), unless stated otherwise. The liquid chromatographic mobile phase consisted of double quartzglass distilled water and acetonitrile (Lichrosolv grade, Merck) in the amounts of 30 and 70 vol.% resp.. To the water 0.01 % amine modifier I (Natec, Hamburg, G.F.R.), which is 2,4 N-di(2-ethylamino)diaminobutane, $NH_2(CH_2)_2NH(CH_2)_4NH\ (CH_2)_2NH_2$, was added. The 2-cyanoacetamide reagent was prepared in a buffer solution of pH = 7.35 containing 32 mmole l^{-1} sodium tetraborate and 66 mmole l^{-1} sodium dihydrogen phosphate. To this buffer 20 mmole l^{-1} 2-cyanoacetamide was added. The pH of the buffer was varied by adding different amounts of the sodium tetraborate and sodium dihydrogen phosphate.

RESULTS AND DISCUSSION

The separation of carbohydrates was performed on a silicagel column physically modified with a polyfunctional amine (7), water acetonitrile mixtures being used as mobile phases. A recent review on the analysis of carbohydrates by column liquid chromatography is that by Verhaar and Kuster (8). The reaction was carried out in a coiled tubular reactor because this type of reactor can be used in an alkaline medium at elevated temperatures, while a packed bed reactor filled with glass beads may cause difficulties at pH values higher than 7.0.

The reaction time was chosen to be 60 s. A short capillary tube was used to cool the reaction mixture before it entered the fluorometric flow cell and to prevent the mixture from boiling.

This capillary tube thus acted as a cooler and as a back pressure device. The low temperature enhances fluorometric sensitivity; at elevated temperatures the signal is strongly quenched.

Optimization of the reaction conditions

The yield of the reaction can be increased considerably by heating the reaction mixture (9). Figure 2 shows the fluorescence intensity of the reaction product of 2-cyanoacetamide and glucose as a function of the reactor temperature at a constant residence time in the reactor. The gain is about a factor of 10 if 150 °C is applied instead of 100 °C, as was done by Honda et al (5,10). Even higher temperatures can be applied but as for the sake of easy handling 150 °C was chosen. As shown by Honda et al (5) the pH dependence of the reaction is of importance for optimization purpose. Figure 3 illustrates the pH influence on the reaction yield and thus on the fluorescence response for glucose at 150 °C. The pH of the reagent solution was measured at room temperature and the optimum was found at the value of 7.35 for a tetraborate/phosphate buffer. For a borate/boric acid buffer the optimum conditions are found to be pH = 9.30. These conditions are less favourable because of solubility problems, although the same sensitivity is obtained.

Band broadening

In a properly designed post-column reactor the additional band broadening should be as low as possible to reduce the inevi-

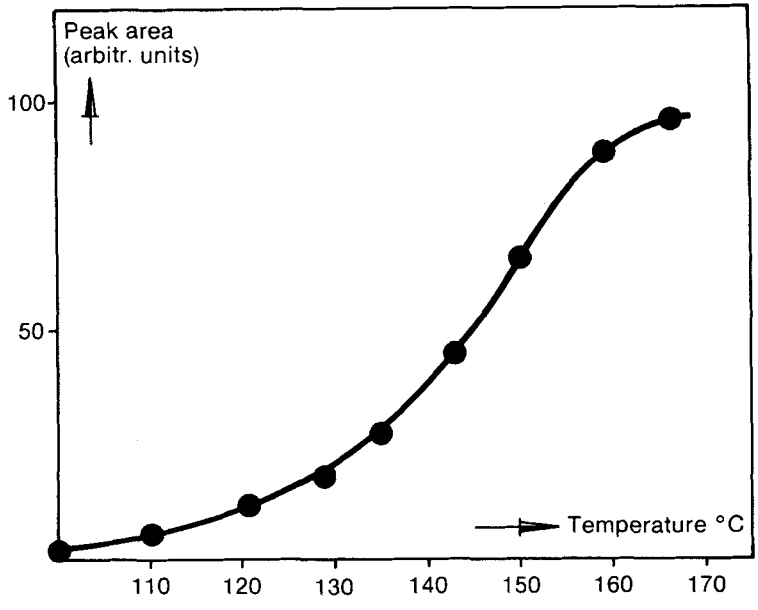

FIGURE 2

Influence of the temperature on the reaction measured by the fluorescence intensity of the reaction product of 2-cyanocetamide and glucose. The residence time is 60 s and the pH of the reaction mixture at room temperature is 7.35.

table loss in resolution (11). In this study we paid attention to this subject also. The variance of the chromatographic peaks was measured and calculated by an interactive procedure (6) by which the second order central moment is determined from the zeroth and first moments. The additional band broadening contribution of each individual part of the liquid chromatographic equipment was measured: in constructing the system, one component was added at a time and the increase in total variance due to the addition of each component was measured. This was done by injecting the reac-

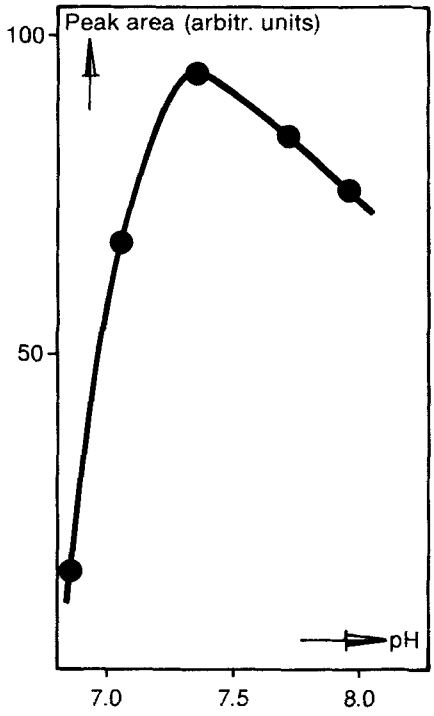

FIGURE 3

Influence of pH of the reaction medium on the reaction yield determined by measuring fluorescence intensity of the reaction product of 2-cyanoacetamide and glucose. Reactor temperature: 150 °C.

tion product of 2-cyanoacetamide and glucose, which had been prepared separately by refluxing 120 ml of reagent solution and 50 mg of glucose for 20 hours, into the system, from which the separation column had been deleted. The results of these experiments are summarized in Table 1. It is clear that the contribution of the reactor is small in comparison to the 15 m long cooler. This can be explained by the difference in diffusion coefficient of the

TABLE 1

Experimental determination of the band broadening in the reaction detector for carbohydrates.

	σ_t^2, s^2
injector + fluorometer	3.4
reactor 20 m x 0.25 mm ID, 150 °C	2.0
cooler 15 m x 0.25 mm ID, 20 °C	13.9
cooler 2 m x 0.10 mm ID, 20 °C	1.0

carbohydrate or the product formed (1) in the reactor effluent at 150 and 20 °C. The miniaturized cooler of 2 m in length and 0.10 mm ID is to be preferred for its lower level of additional band broadening. The total variance in time units of the reaction detection system becomes 6.3 s^2 when the 2 m long cooler is used, which is only a minor contribution to the overall variance (including the separation column) of 40 s^2. Resolution of the components and overall efficiency (the HETP value of fructose is 26 μm) are only little influenced by this extra-column band broadening. The separation of a test mixture is illustrated in Figure 4. Although a reaction detection system was used the resolution of fructose and glucose is good and even better than with several separation systems on polystyrene-divinylbenzene packing materials as presented in the literature.

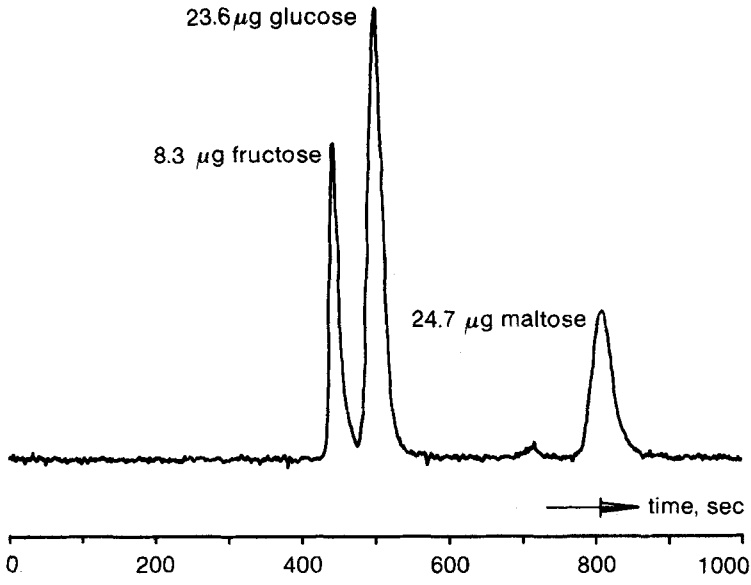

FIGURE 4

Chromatogram of a test mixture of fructose, glucose and maltose.

Quantitative analysis

Quantitative evaluation of the chromatographic data is generally done by the external standard method. In order to find the linear dynamic range of a method and the detection limits, calibration curves are measured. Calibration curves of the chromatographic method using reaction detection are shown in Figure 5. For glucose and fructose a linear relationship is obtained; all datapoints are average values of duplicate measurements.

The precision of the analytical method is determined by at least 5 consecutive injections of standard test mixtures at several

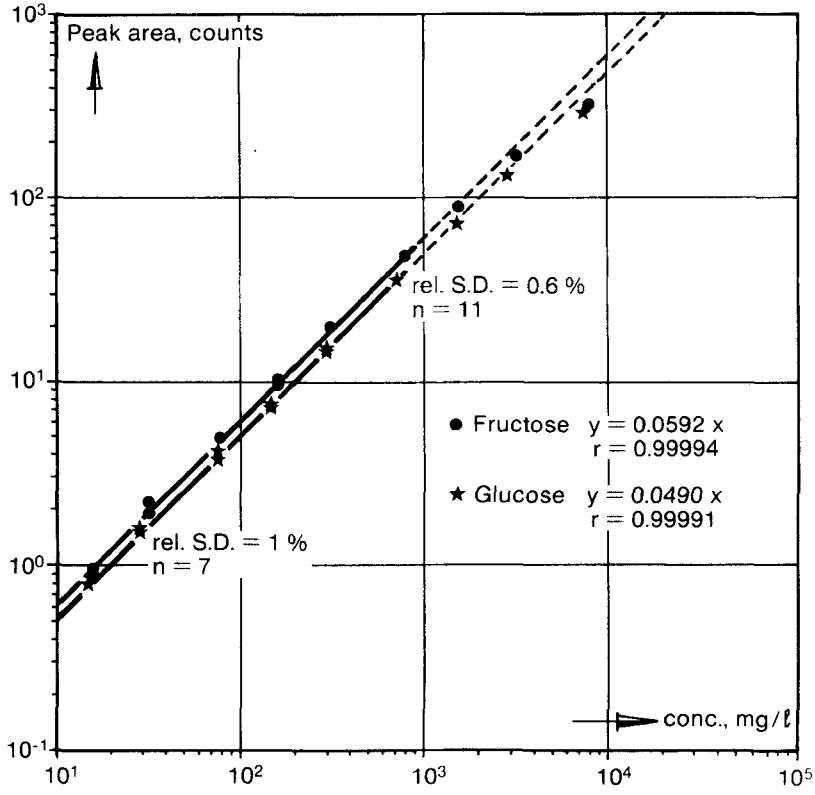

FIGURE 5

Calibration curves for glucose and fructose using fluorometric reaction detection with 2-cyanoacetamide.

concentration levels and calculation of the relative standard deviation (rel. S.D.) of the response factors. As indicated in Figure 5 a relative standard deviation better than 1 % can be achieved even for a reaction detection system as described. This is confirmed by earlier studies (12). For comparison reasons the calibration curves of fructose and glucose are determined with a refractive index detector too (Figure 6). A good linearity over

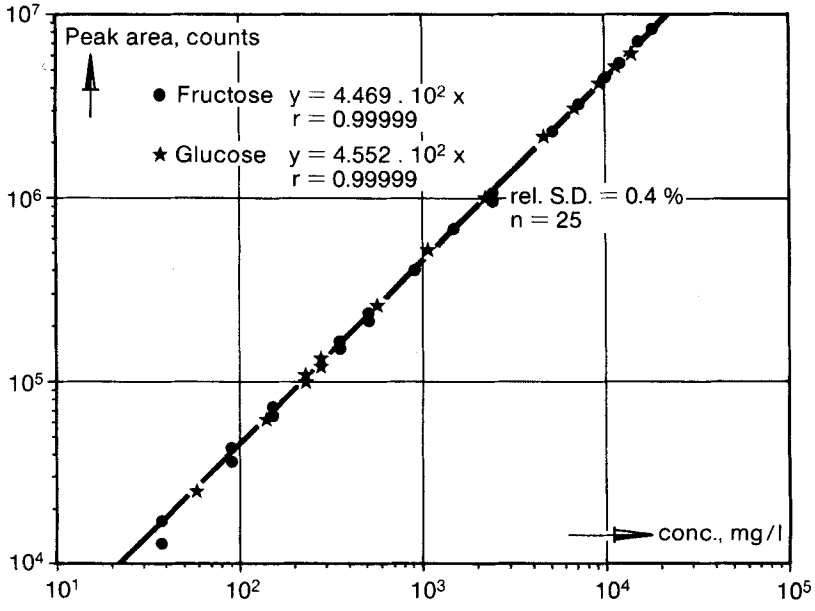

FIGURE 6

Calibration curves for glucose and fructose using differential refractive index detection.

3 decades was obtained. The refractive index detector has the same detection limit (10 mg l^{-1} for fructose) as the reaction detection fluorometry and a wider linear dynamic range. The use of a excitation as well as emission variable wavelength fluorometer would be preferable in order to use at the maximum excitation and emission wavelengths of 331 nm and 383 nm respectively. Moreover no cut-off filters and bandpass filters, which reduce light intensity, need to be used. Besides the concentration of 2-cyanoacetamide is restricted because of limited solubility in water/acetonitrile mixtures. On the other hand the fluorometric reaction detection

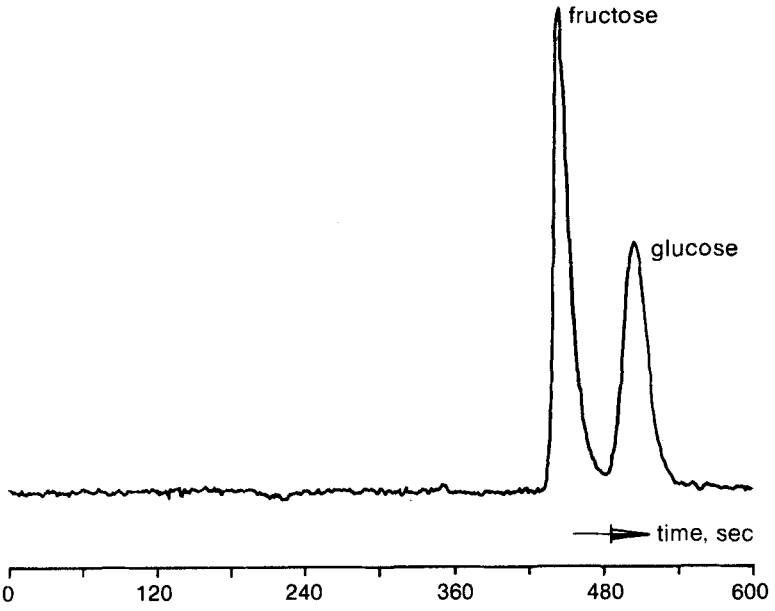

FIGURE 7

Chromatogram of honey containing 41 wt.% fructose and 41 wt.% glucose (a solution of 0.09 wt.% honey in water was injected).

method shows a higher detection selectivity, which is illustrated by the chromatogram of honey in Figure 7. Of the numerous constituents of honey, only fructose and glucose are detected.

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