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GAS–LIQUID CHROMATOGRAPHY OF MONOSACCHARIDES AT THE PICOGRAM LEVEL USING GLASS CAPILLARY COLUMNS, TRIFLUOROACETYL DERIVATIZATION AND ELECTRON-CAPTURE DETECTION

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SUMMARY

A gas–liquid chromatographic (GLC) method has been developed for the analysis of samples containing nanogram to picogram amounts of three different types of monosaccharides, *viz.*, aldoses, ketoses and deoxy sugars. The minimum detectable amounts were at the sub-picogram level when highly electron-capturing derivatives such as trifluoroacetyl (TFA) esters of sugars were used. The gas chromatograph was modified to utilize the powerful combination of a glass capillary column and an electron-capture detector.

For nine monosaccharides all of the anomers were resolved on a glass capillary column with particularly good deactivation properties. The losses of the TFA derivatives on the column were determined approximately at different carrier gas flow-rates. Quantitative determination was facilitated by taking all anomeric forms into account.

The sensitivity and resolution of the method permit extremely small amounts of sugars in a complex mixture to be studied. This has been demonstrated on a sea-water sample.

INTRODUCTION

Common methods for the quantitative determination of individual free monosaccharides are based on liquid chromatography using ion-exchange resins and colorimetric determination of the usually well separated peaks[1,2]. Monosaccharides can also be determined by gas–liquid chromatography (GLC) after the formation of their O-methyl, O-acetyl, O-trifluoroacetyl (TFA) and O-trimethylsilyl (TMS) derivatives. The separation of the monosaccharides by liquid chromatography has the important advantage that it is not affected by the mutarotation equilibria in aqueous solutions. However, GLC has certain advantages when a large number of samples containing small amounts of sugars need to be analyzed in reasonably short time.

The main difficulty in the GLC separation of different monosaccharide derivatives is the formation of two to four peaks per monosaccharide. These peaks result from anomeric and ring isomerization (pyranose and furanose rings). In a complex mixture containing a number of sugars, the multiplicity of peaks produced will require

an extremely high efficiency of separation of derivatives that have very similar structures. An alternative method is to reduce the sugars to the corresponding alcohols, followed by derivatization which yields only a single peak as a result of the destruction of the ring structures[3]. However, these alcohols will not distinguish between certain pairs of sugars, as sugar pairs such as lyxose–arabinose and gulose–glucose will give the same alcohol upon reduction. When ketoses and aldoses, *e.g.*, sorbose and glucose, are present in the same sample they will also give the same alcohol, glucitol. Ketoses always give two different products upon reduction, *e.g.*, fructose produces both mannitol and glucitol. It should also be pointed out that Wohl derivatives do not give volatile products of ketoses, which limits their use[4].

Attempts have been made to obtain very high resolutions on GLC columns, thereby separating all the anomers of the monosaccharide derivatives. Zanetta *et al.*[5] showed that a single analysis on a 2-m packed column was sufficient to resolve a mixture of TFA derivatives of the O-methylglycosides, but the corresponding simple TFA monosaccharides could not be resolved. The sugars could still be quantitatively determined by taking into account the relative proportions of the isomers at equilibrium in water.

Tesarik[6] found that use of wall-coated open-tubular glass columns gave a more complete separation of the different TMS sugars. The use of two capillary columns with stationary phases of different polarity allowed the identification of 11 sugars in a mixture.

Very high sensitivity has been achieved by making sugar derivatives with a high electron-capture response. A method for the determination of sugars at the nanogram level was reported by Tamura and Imanari[7], who used TFA derivatives and electron-capture detection. Monosaccharides, which as TFA derivatives have up to 15 fluorine atoms incorporated, will have good electron-capture properties.

The combination of the high resolving power of wall-coated open-tubular glass columns and the extreme sensitivity of the electron-capture detector gives many new advantages in the analysis of complex mixtures. Franken and co-workers[8,9] studied pesticides and derivatized biological amines by using this arrangement. Schulte and Acker[10] studied the PCBs with the combination of a glass capillary column and an electron-capture detector. Recent papers[11,12] have described some of the problems that arise when connecting the columns to the electron-capture detector, which has a fairly large dead volume compared with the carrier gas flow-rate.

The purpose of this work was to develop a method for the GLC analysis of a complex mixture of monosaccharides in sea-water samples. The free neutral sugar content in sea water is low and the concentration of individual saccharides is at the micrograms per litre level[13]. With liquid chromatography it was necessary to desalinate and evaporate 0.5–1 l of sea water, which is a time-consuming procedure. With a more sensitive chromatographic method, the volume of the sea-water sample could be considerably reduced.

EXPERIMENTAL

Apparatus

A Perkin-Elmer 3920 gas chromatograph equipped with a nickel-63 electron-capture detector was used. The gas chromatograph was modified to suit glass capillary

columns. Problems that must be solved when using glass capillary columns are dead volumes in the injector and in the connections of the column, manifold and electron-capture detector, which have to be avoided in order to minimize band broadening and loss of chromatographic resolution.

The injector was modified to a Grob-type injector[14]. In addition to a split flow, this type of injector also has a flush flow that purges the septum and injector volumes. The glass liner in the injector was replaced with one of I.D. 1 mm, thus decreasing the dead volume in the injector. Fittings without dead volumes were installed for connecting the column. At the column end, a scavenger gas (argon plus 5% of methane) was added in order to minimize band broadening in the manifold and the detector. The flow-rate of the scavenger gas was 24 ml/min. In order to obtain a complete all-glass system, the steel tubing connecting the glass capillary column and the electron-capture detector was replaced with glass-lined steel tubing.

All chromatograms were recorded on a Perkin-Elmer 56 recorder.

Materials

The monosaccharides were purchased from Merck (Darmstadt, G.F.R.) (Vergleichsubstanzen für die Papierchromatographie).

Dichloromethane of pro analysi quality was used. Purum-grade *n*-hexane was distilled over phosphorus pentoxide on a 10-plate bubble-cap column. Spectra-analyzed isooctane (Fisher Scientific, Pittsburgh, Pa., U.S.A.) and trifluoroacetic anhydride (TFAA), 99% pure (Pierce, Rockford, Ill., U.S.A.) packed in glass vials under nitrogen and anhydrous conditions were used.

For testing other derivatives we used heptafluorobutyric anhydride (HFBA) (Pierce) and pentafluoropropionic anhydride (PFPA) (Pierce).

Prior to use, the methylene chloride, *n*-hexane and isooctane were dried over magnesium sulphate, which had been heated at 200° overnight.

Derivatization

TFA derivatives. Standard aqueous solutions of nine monosaccharides (rhamnose, arabinose, xylose, ribose, glucose, fructose, mannose, galactose and lyxose) were prepared. The solutions were preserved by adding a few drops of a saturated aqueous solution of mercury(II) chloride. Lyxose was chosen as the internal standard as it is rare in nature. The other sugars are common in sea water[13]. The concentrations of the standard solutions ranged between 101 and 148 μg/ml. Aliquots of the standard solutions were transferred into 1-ml glass vials (Reacti-Vials, Pierce) with a 10-μl Hamilton syringe. After freeze-drying, 100 μl of TFAA and 100 μl of dichloromethane were added with a 100-μl Hamilton syringe under a stream of dry nitrogen. The vials were then tightly closed with PTFE-lined screw-caps and heated in an oil-bath at 130° for 2 h. After cooling, the reaction mixture was evaporated under dry nitrogen with gentle heating from an IR lamp to a volume of approximately 10 μl. Then 100 μl of dry *n*-hexane containing 1% of TFAA were added and the mixture was again evaporated down to about 10 μl. Finally, after adding a further 100 μl of *n*-hexane–TFAA mixture, the solution was ready for injection into the gas chromatograph.

For splitless injection, the *n*-hexane was replaced with isooctane in order to

achieve the solvent effect described by Grob and Grob[15], in which the solvent participates and improves the chromatographic separation process.

Other derivatives. Standard aqueous solutions were freeze-dried as described above and 100 μl of PFPA or HFBA were added. No solvent was used during the derivatization step. The reaction mixture was heated at 70° for 2 h. After cooling, the reaction mixture was evaporated to a volume of approximately 10 μl and 100 μl of dry *n*-hexane were added. The mixture was again evaporated down to about 10 μl and 100 μl of dry *n*-hexane were added before injection into the gas chromatograph.

Gas chromatographic procedure

The general conditions were as follows: helium carrier gas flow-rate, 0.79 ml/min; splitting ratio, 1:12.5; temperature programme, 70–130° at 1°/min; injection temperature, 200°; detector temperature, 250°; manifold temperature, 200°.

For splitless injections we used the following temperature programme: 74° isothermal for 4 min, then increased at 1°/min up to 130°. The splitless period was 1 min.

For determination of the minimum detectable amount the carrier gas flow-rate was 0.77 ml/min, splitting ratio 1:80 and column temperature 100° (isothermal).

Preparation of the sea-water samples

The sea-water samples were desalted by ion-exchange membrane electrodialysis as described by Josefsson[13] and Hirayama[16]. A commercially available electrodialysis cell (Shandon) with a sample volume of 40 ml was used. The membranes were Permaplex C-20 and A-20 (Permutit). The current applied over the electrodes was 600 mA at the start. The desalting time for a 40-ml sea-water sample with a salinity of 3.5% was about 3 h.

RESULTS AND DISCUSSION

Derivatization

Different workers have reported various reaction conditions and solvents for producing TFA derivatives of monosaccharides[5,7,17]. The strong electron-capturing properties of the reagents and their leaving groups make it necessary to remove them from the reaction mixture before analysis, otherwise the detector may be overloaded. The choice of solvent is important as it has to be free from contaminants. At the same time, the equilibrium of the anomers from the dried aqueous solution should not be changed by solvent effects before the formation of the derivatives[3]. We found that solvents such as acetonitrile, tetrahydrofuran and pyridine could not be used for these reasons. Zanetta *et al.*[5] proposed dichloromethane without any catalyst as a suitable solvent. This solvent can easily be evaporated with the remaining reaction products TFAA (b.p. 40°) and TFA (b.p. 74°) without any loss of sugar derivatives. The optimal derivatization conditions were found to be 130° and 2 h without any added catalyst such as triethylamine or pyridine.

Heptafluorobutyric anhydride was proposed by Brooks *et al.*[18] for esterification of hydroxyl groups. We found, when testing HFBA and PFPA reagents for derivatization of monosaccharides, that the remaining reagent and leaving groups with high boiling points were difficult to remove without losses of sugar derivatives. The HFB

and PFPA derivatives gave 5–6 times higher responses than the TFA analogues. However, it was very difficult to analyse PFP and HFB derivatives, because after some injections a broad peak appeared, which was a result of the remaining acids (b.p. 97° and 120°) in the column.

Glass capillary columns

The separation of sub-nanogram amounts of TFA derivatives of monosaccharides requires glass capillary columns of very high quality. In a column with inadequate deactivation of the glass surface the sugar derivatives are easily adsorbed and/or decomposed.

We have investigated several published methods for the preparation of glass capillary columns in order to find a method that gave columns suitable for separation of TFA monosaccharides.

Some workers[19,20] have suggested etching of Pyrex glass with hydrofluoric acid as a useful surface pre-treatment, followed either by deactivation with TMCS and HMDS in the gas phase[21] or by the Carbowax treatment described by Cronin[22]. In our experience, these etching procedures will produce columns with high separation efficiencies. The efficiency (expressed as coating efficiency) lies between 60 and 80%, with no significant difference between the methods. It was not possible, however, to

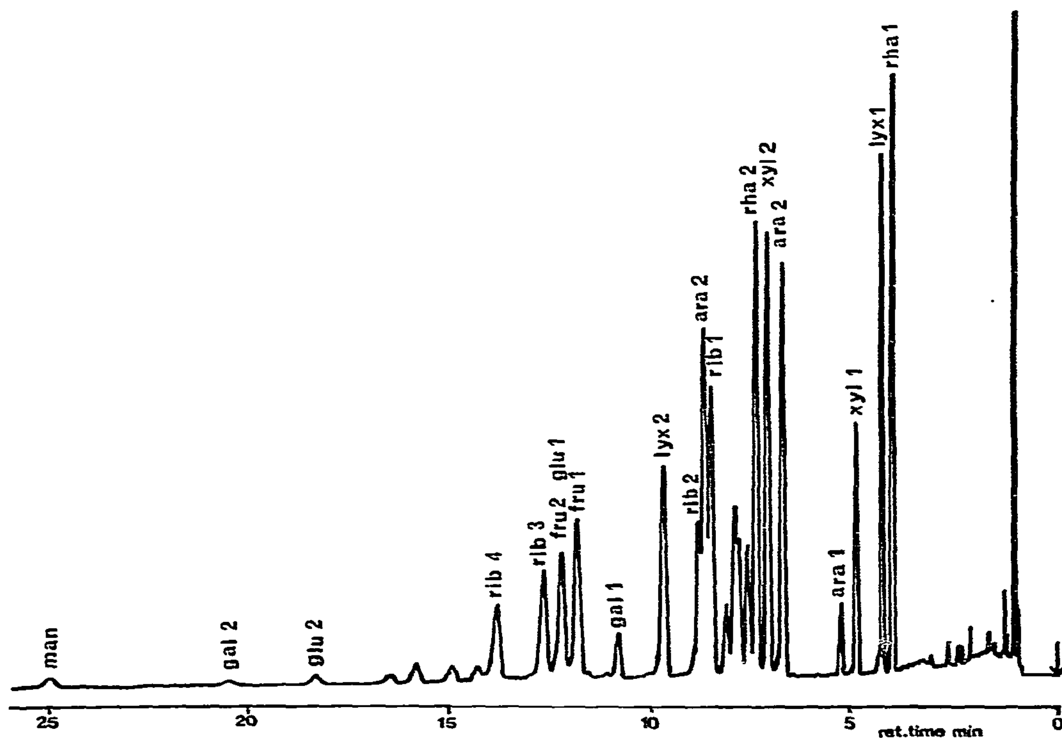

Fig. 1. Chromatogram of nine monosaccharides as their TFA derivatives; approximately 50 pg of each on the column. Stationary phase, OV-101; column, 18 m × 0.3 mm I.D.; temperature, 85° (isothermal). Rha = rhamnose; lyx = lyxose; xyl = xylose; ara = arabinose; rib = ribose; glu = glucose; fru = fructose; gal = galactose; man = mannose.

analyse sub-nanogram amounts of TFA monosaccharides on these columns. The derivatives either vanished or were eluted with severe tailing. We attribute this effect to the inadequately deactivated surface of the etched glass.

We have also investigated a procedure for the preparation of glass capillary columns recently published by Grob and Grob[23]. In this method, surface roughening is accomplished by deposition of very fine crystals of barium carbonate on the glass surface. The surface is then deactivated with repeated treatments with Carbowax 20M and Emulphor ON 870. This method will also produce glass capillary columns with high coating efficiencies (60–80%). In contrast to the etched columns, it was possible to analyse TFA monosaccharides at the picogram level on these barium carbonate treated columns (see Fig. 1).

We have also tested several commercially available glass capillary columns. A 50-m SE-54 column purchased from G.C. Labor (H. & I. Jaeggi, Trogen, Switzerland) was the only one that gave acceptable results (Fig. 2).

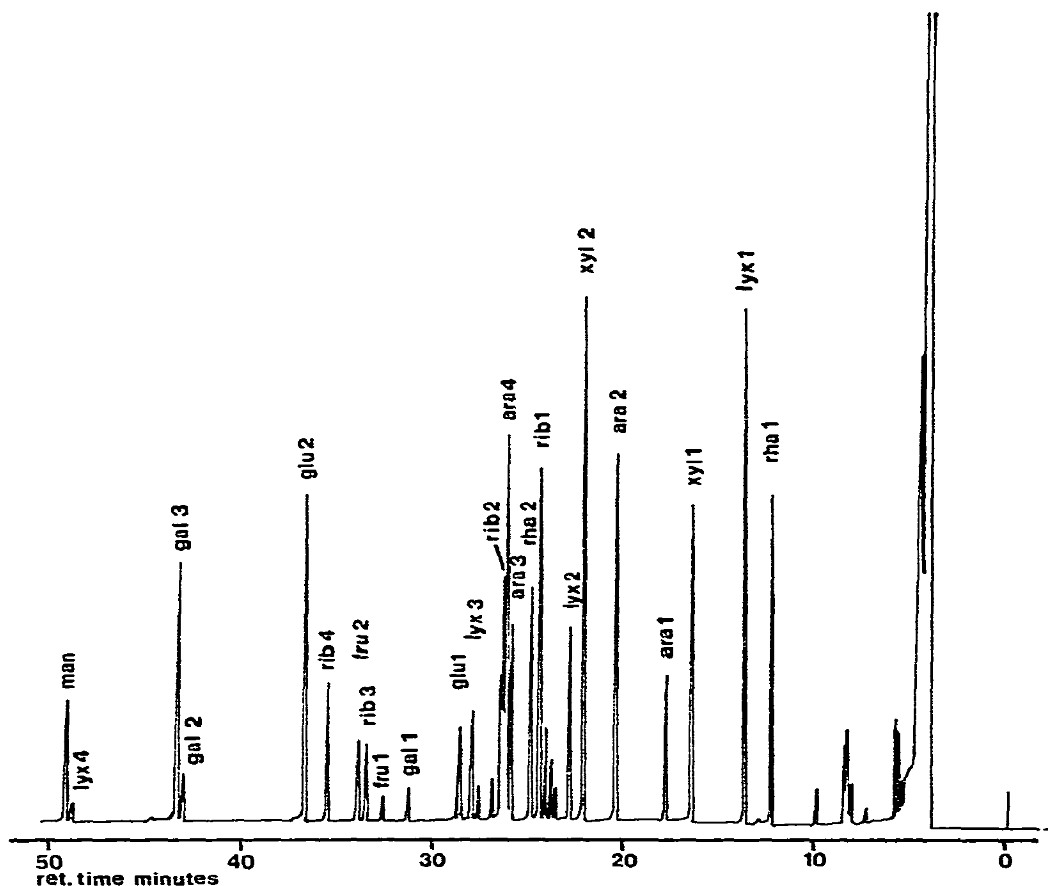

Fig. 2. Chromatogram of nine monosaccharides as their TFA derivatives; approximately 1 ng of each on the column. Carrier gas flow-rate, 0.79 ml/min; scavenger gas flow-rate, 24 ml/min; temperature programme, 74° (4 min) to 130° at 1°/min; stationary phase, SE-54; column, 50 m × 0.34 mm I.D.; 1 μl injected, splitless. Notations as in Fig. 1.

Adsorption and decomposition of TFA derivatives on the column

The decomposition and deposition of TFA derivatives of monosaccharides were studied by Jansen and Baglan[24]. They found that about 95% of a ^{14}C-labelled TFA-glucose decomposed or deposited in a packed column. The loss of the derivative was not found to be temperature dependent. In our experience, decomposition and deposition of TFA derivatives of sugars is indeed a serious problem, and we tried to quantify the loss of TFA derivatives as follows. TFA-glucose-2 (1 ng), with dichlorobenzene as internal standard, was analysed at different carrier gas flow-rates, thus varying the hold-up time in the column. Dichlorobenzene is assumed not to decompose or adsorb in the column.

The peak heights of TFA-glucose-2 and dichlorobenzene were plotted against the retention time (see Fig. 3). The ratio between the peak heights for TFA-glucose-2

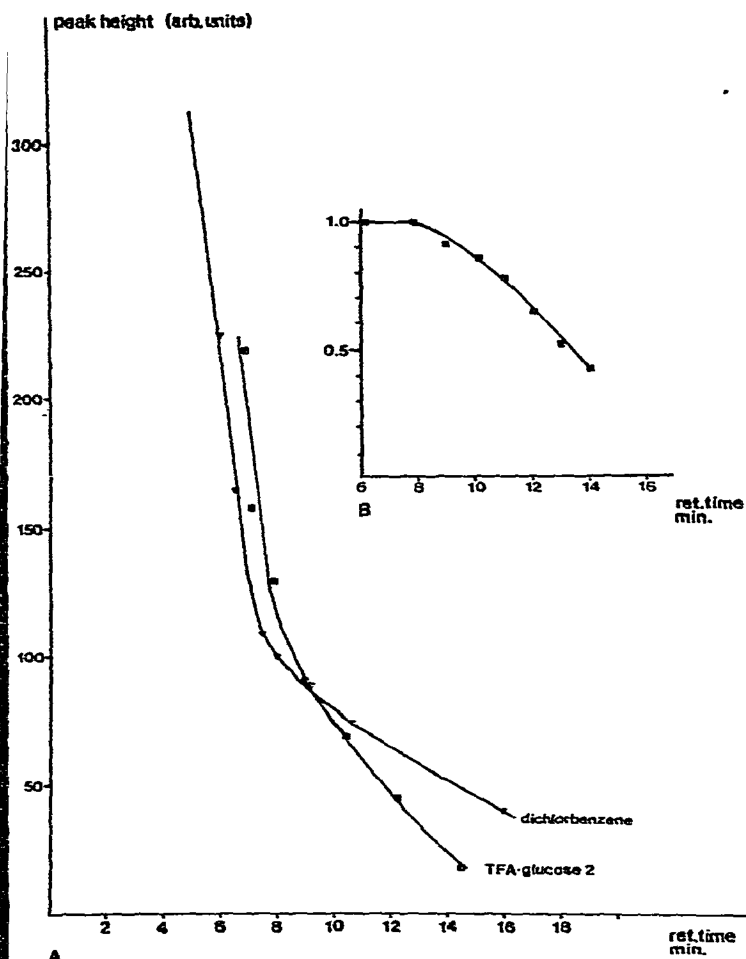

Fig. 3. Approximate fraction of TFA-glucose-2 that is lost on a 50-m SE-54 column compared with dichlorobenzene (inset). This graph was obtained by varying the carrier gas flow-rate and measuring the peak heights of dichlorobenzene and TFA-glucose-2.

and dichlorobenzene for a given retention time was then plotted against the retention time (see Fig. 3). This plot gives the approximate fraction of the sample that is not lost in the column. As can be seen from Fig. 3, more than 50% of the sample was lost at retention times larger than 14 min. In order to correct the results for these column effects, it is essential to use an internal standard with properties similar to those of the sample.

Quantitative determination

The resolution of all isomers of the nine sugars was sufficient for a quantitative determination of the main monosaccharides in sea water, using lyxose as internal standard. For quantitative purposes it is an advantage to measure the peak heights of all anomers, as the mutarotation equilibrium in water can be shifted when changing to another solvent in the derivatization step[3].

Calibration graphs are constructed by plotting the sum of the peak heights of the anomers of each monosaccharide divided by the sum of peak heights for the anomers of lyxose against the amount of the corresponding monosaccharide, as can be seen in Fig. 4. The amount injected on the column ranged between 0.2 and 1.5 ng, and the total amount of each monosaccharide that had been derivatized ranged between 0.2 and 1.5 μg. The four graphs represent four different types of sugar, *viz.*, aldohexoses (glucose), ketohexoses (fructose), aldopentoses (arabinose) and deoxy-

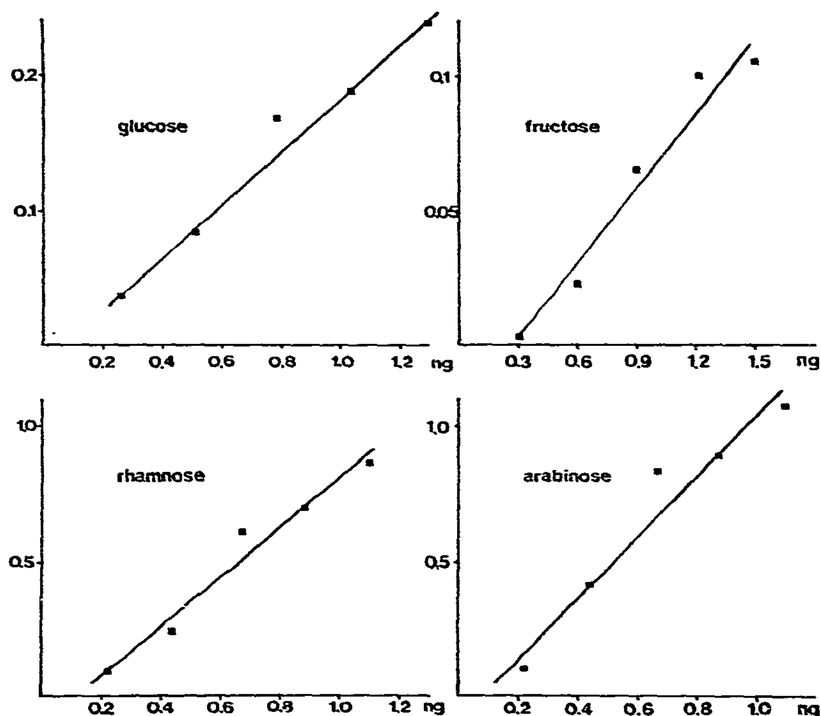

Fig. 4. GLC response plots in the picogram range for glucose, fructose, rhamnose and arabinose. The sum of the peak heights for each monosaccharide divided by the sum of the peak heights for lyxose (used as internal standard) is plotted against the amount of monosaccharide injected on the column.

TABLE I

REPRODUCIBILITY FOR THE DIFFERENT MONOSACCHARIDES

Standard deviations are given relative to the internal standard.

TFA derivative	*Relative standard deviation*		
	Derivatization (12 different samples)	*Gas chromatography (same sample)*	
		Split 1:12.5 (3 times)	*Splitless (4 times)*
Mannose	18	5	11
Xylose	10	8	17
Ribose	21	1	7
Rhamnose	16	4	2
Arabinose	27	4	17
Fructose	30	6	12
Galactose	9	4	13
Glucose	9	2	13

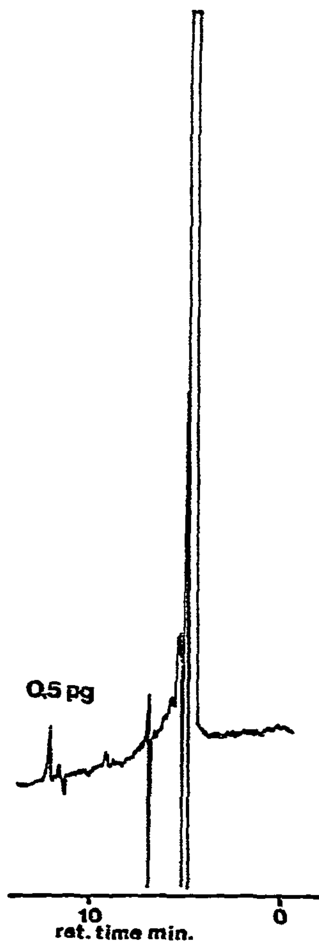

Fig. 5. Chromatogram showing the minimum detectable amount of TFA-rhamnose. Approximately 0.5 pg on the column and a carrier gas flow-rate of 0.77 ml/min. Column temperature, 90° (isothermal).

aldoses (rhamnose). The GLC response plots showed less scatter for glucose, arabinose and rhamnose but were about the same for fructose. Ketoses such as fructose are not as stable as the other sugars. The relative standard deviation for the whole method lies in the range 10–30% for the different sugars (see Table I).

The application of splitless injection has been reported[14] to give a decreased injection error, but in this instance the error increased, as can be seen in Table I. Our explanation is that the injector volume was not optimally designed for this analysis.

Limit of detection

A chromatogram obtained on analysis of TFA-rhamnose is presented in Fig. 5, showing the detection of 0.5 pg injected with a splitting ratio of 1:80 and a carrier gas flow-rate of 0.77 ml/min. The minimum detectable amounts for the different monosaccharides were of the same order. The effect of the carrier gas flow-rate or the retention time may alter the response considerably, which was illustrated in Fig. 3.

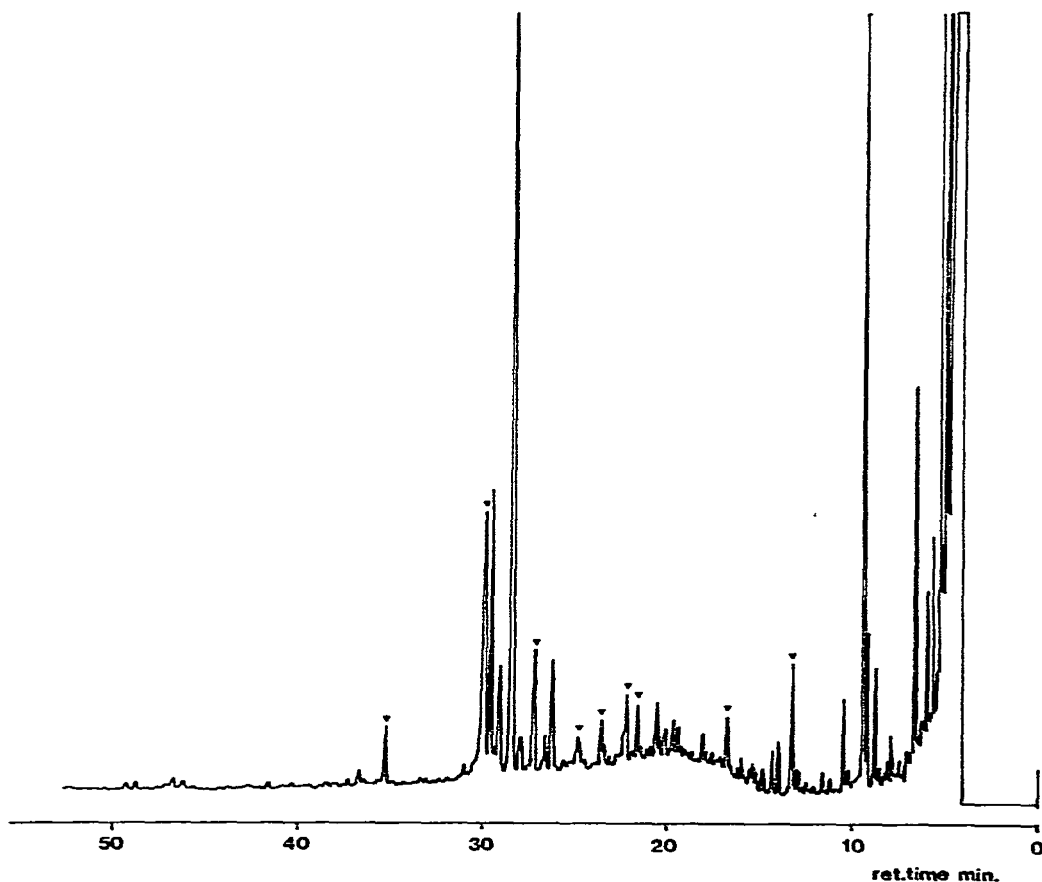

Fig. 6. Chromatogram of sea-water sample. Monosaccharides that can be identified from our standard are marked with arrows.

Sea-water sample

The chromatogram from a sea-water sample is presented in Fig. 6. The desalting procedure removes all materials besides neutral organic compounds which are not affected by the electrical field. Thus neutral sugars, glycols and similar compounds remain in the desalinated sample. The chromatogram shows that there are compounds present other than the studied monosaccharides. Fucose, sorbose and some glycols are probably present, and have been reported earlier to exist in sea water[13]. A more detailed investigation on the carbohydrate composition in sea water will be reported later.

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