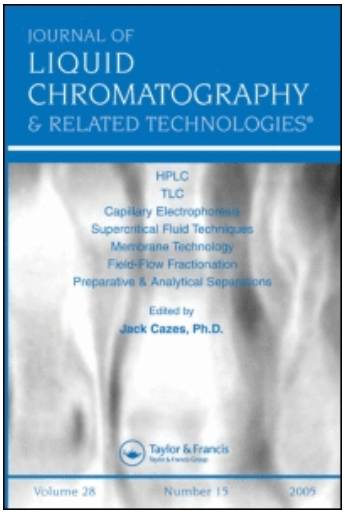

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Rapid HPLC Methods for the Separation and Quantitation of a Mono-, Di-, and Tri-Saccharides Mixture and Applications

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RAPID HPLC METHODS FOR THE SEPARATION AND QUANTITATION OF A MONO-, DI-, AND TRI-SACCHARIDES MIXTURE AND APPLICATIONS.

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ABSTRACT

A simple, rapid method has been developed for the separation and quantitation of mono-,di-, and tri-saccharides. The method utilizes a 30cmx 3.9mm i.d. Microbondapak NH_2 column, refractive index detection and water-acetonitrile elution. Two chromatographic systems are described. The isocratic mode was necessary to develop a procedure.20 Carbohydrate's retention times were evaluated. To optimize the separation of nine water-soluble sugars , a gradient mode flow-programming was used. Separation was achieved within 28 minutes. The low detection limit (4 micrograms) of the above chromatographic procedure and its different possibilities could be of great interest to the analyst. The method has been successfully applied to quantify the major carbohydrates found in two types of commercial honey.

INTRODUCTION

There are mainly two techniques for the separati and quantitation of carbohydrates in HPLC.

Derivatized sugars, soluble in the non-polar solvents and detected by UV absorption at an appropriate wavelength; water-soluble sugars which can be directly eluted, without pretreatment, with colorimetric, refractive index and/or UV absorption detections.

As pointed out so often, the gain in sensitivity and the resolving power are advantages of the first method. Isomerization can even be avoided by working under extremely rigorous conditions (1). However, the derivatization is cumbersome; derivative preparation is time-consuming, and derivative recovery is seldom quantitative. Moreover, other physico-chemical methods of analysis are needed to identify the by-products which very often result from derivatization.

For the second method, numerous modes of HPLC have been investigated and reported in the literature. Ion-xchange columns for HPLC have been evaluated (2); they equire long chromatographic runs. The trend to fast eparations in carbohydrate analysis can be seen from ue increasing use of siloxane-bonded phases as statio-ry phase.

e most widely used stationary phase is an amino-propyl oup bonded to silicagel (3-8). Besides this amino phase, no and combinations of amino and cyano have been used cessfully (9). With the moderately polar stationary ees, the most suitable polar mobile phase remains

water-acetonitrile in different ratios. In these chromatographic systems, the carbohydrates are analyzed without prior chemical transformation . The means of detections usually employed are : refractive index, and UV detection at 188 nm (10) or at 192 nm (11).

The aim of this work was to study the chromatographic behaviour of a range of mono-, di-, and tri-saccharides on a microbondapak NH_2 column with refractive index detection. The separation and quantitation were also evaluated to determine the major sugars contained in two types of Algerian honeys.

MATERIALS

Reagents :

The acetonitrile, spectrophotometric grade, was obtained from E. Merck, Darmstadt (GFR), and Eastman (USA);acetic acid from E. Merck; sugars and sugars alcohols are reference substances for chromatography, purchased from E. Merck.

The studied honeys were available from Sogedia(ALGERIA). The standards were prepared by dissolving amounts of carbohydrates in distilled water in the range 20-100 mg/ml (2-10%w/v). The weighed honey samples were diluted with water-acetonitrile mixture (50/46 v/v), filtered and injected directly into the chromatograph.

Apparatus :

Chromatograms were run on :

a Waters Associates liquid chromatograph model ALC/GPC 244 equipped with two M 6000 solvent delivery systems, a M 660 solvent programmer, a U-6K universal injector and a MR 401 refractive index detector.

Two recorders were employed :

Houston Instruments Omniscribe (Austin, Texas)operated at 10 mV sensitivity (Attenuation : 8x) and a chart speed of 0.1 in/min for separations;

Sefram Servotrace (type PE Paris) used at variable voltage range (Attenuation : 256-16 x) and a chart speed of 0.1 in/min for quantitations (e.g., calibrations curves). The separations were conducted with a 30cm x 3.9mm i.d. Microbondapak NH_2 stainless steel column (Waters Associates). The samples were injected with a 10 microliter syringe (Scientific glass Engineering P.T.Y./Ltd, AUSTRALIA) for separations and 1, 5, 10 and 20 microliter syringes (Hamilton Co., Reno,Nevada) for quantitations. Filtration of carbohydrate standards and honey solutions was carried out using Waters Associates sample clarification kit (0.45 microns pore size,aqueous solvent filtration disk). The solvents were filtered using a Pyrex filter holder; 0.45 microns pore size,type HA of the filter membrane (Millipore Corp., Bedford, Mass.).

METHODS

The chromatographic system was employed in the single-pump isocratic mode for development of a procedure and in the single-pump gradient flow rate mode to establish the best chromatographic conditions for the routine analysis. All chromatographic analyses were carried out at room temperature (ca. 20-22°c). The solvent mixtures were degassed before each assay. After gradient flow-rates, the gradients were reversed for 10 min to reestablish initial conditions. Flow-rates were maintained between 0.8 and 2.5 ml/min. Water-acetonitrile mixtures were used as elution solvent and all quantitations in this report were made on the basis of peak height measurements.

RESULTS AND DISCUSSION

The porous support of the used column is made by chemically bonding an amino group to silicagel microparticles (microporasil) at 9% by weight (12). With this stationary phase, the first approach was to study the polarity effect of the water-acetonitrile mobile phase (by increasing the percentage of water)with a 5-monosaccharide separation. The best elution-with, however, some broad peaks - had been obtained by using the above mobile phase in the ratio 10/90 (Figure 1a). To improve

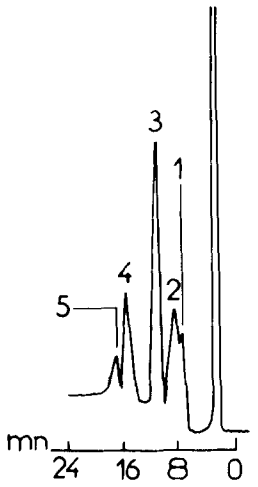

FIGURE Ia : Isocratic mode sepation of 5 monosaccharides: I.D (+)-xylose; 2.L(+)-arabinose; 3.D(-)-fructose; 4.D(+)-glucose; 5.D(+)-galactose. Solvent : Water/Acetonitrile 10/90. Chromatographic conditions were the same as in Table I.

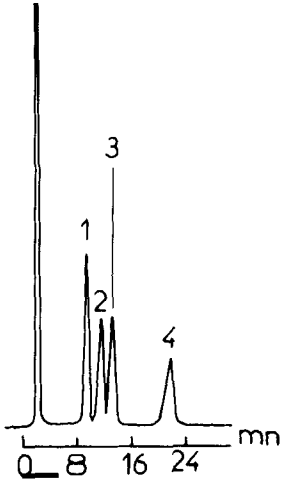

FIGURE 1b : Isocratic mode elution of di-, and tri- saccharides with the same conditions as in Table 2. The peaks 1,2,3 and 4 correspond to : sucrose, maltose, lactose and raffinose respectively.

the peaks symmetry, we refered to TLC.Bolliger and co-workers (13) advise modification, in this case,of the eluting solvent pH by small amounts of buffers such as Na_3PO_4, KH_2PO_4, CH_3COOH, etc.

In the present study, a variable amount of CH_3COOH was added to modify the mobile phase pH. Thereby,the best efficiency and resolution were achieved by acetonitrile-water-acetic acid (90/09/01) elution. With this mixture, 17 carbohydrates were eluted sequentially. Their retention times are indicated in Table 1. We can conclude that the di-,and tri-saccharides are strongly retained by the stationary phase. Indeed, the elution times of these sugars exceed one hour. This same eluting solvent allowed us to separate 6 monosaccharides (Figure 2a) and a fructose-glucose-mannose mixture whose difficulty of separation is often mentioned in the literature (14).The elution of this mixture was carried out by recycling the partially fractionated components (Figure 2b). With regard to di-,and tri-saccharides, they cannot be eluted with reasonable retention times using acetonitrile-water-acetic acid in the ratio 90/9/1. We attribute this fact to the poor solubility of di-and tri-saccharides in the above solvent. This can be improved by increasing the polarity of the mobile phase(i.e. by increasing the water content). After many assays, a satisfactory separation of four di-and tri-saccharides was

TABLE 1

Isocratic Mode Elution of 17 Carbohydrates

a°)Operating Conditions

Column	:	microbondapak NH_2
Attenuation	:	8x
Pressure	:	400 psi.
Solvent	:	$CH_3CN/H_2O/CH_3COOH$ (90/09/01)
Flow-rate	:	1.5 ml / min.
Chart speed	:	0.1 in/mn.
Injections	:	10 microliters of each of samples at 10%(w/v).

b°)Retention Times :

Compound	Retention Times (min.)
2-Desoxy-D-ribose	4.4
L(+)-Rhamnose	6.2
D(-)-Ribose	6.2
Meso-Erythritol	7.2
D(+)-Xylose	8.2
L(+)-Arabinose	9.2
L(-)-Sorbose	11.8
D(-)-Fructose	11.8
D(+)-Mannose	14.6
D(+)-Glucose	16.5
D(-)-Sorbitol	22.5
D(+)-Galactose	17.8
D(-)-Mannitol	23.4
Meso-Inositol	61.3 (peak tailing)
Sucrose	59.3 (peak tailing)
Cellobiose	
Maltose	83.5 (peak tailing)
Trehalose	
Lactose	90.8 (peak tailing)
Raffinose	

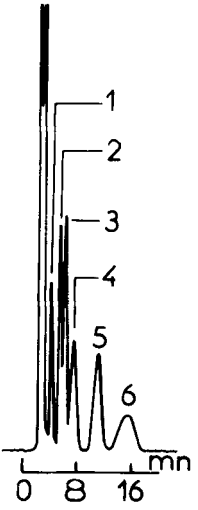

FIGURE 2a : Separation of monosaccharides under isocratic conditions of Table 1; 1.2-deoxy-D-ribose; 2.D(-)-ribose; 3.meso-erythritol; 4.D(+)-xylose; 5.D(-)-fructose; 6.D(+)-glucose.

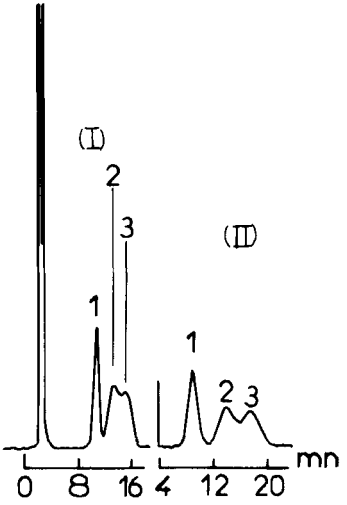

FIGURE 2b : Elution of a fructose (1)-mannose (2)-glucose (3) mixture (I). Elution profile of the same mixture by recycling the mobile phase (first passage), 4 mn after injection of the sample (II). Other conditions were as in Table I.

TABLE 2

Isocratic Mode Elution of Di-,and Tri-saccharides

a°)Operating Conditions :

Column : Microbondapak NH_2
Attenuation: 8x
Pressure : 420 psi.
Flow-rate : 1.5 ml/min.
Chart Speed: 0.1 in/min.
Solvent : water-acetonitrile (20/80).
Injections : 5 microliters of each of samples at 10%(w/v).

b°)Retention Times :

Compound	Retention Times (min.)
Sucrose	9.6
Cellobiose	11.8
Maltose	11.7
Trehalose	12.8
Lactose	13.6
Raffinose	22.0

obtained with water-acetonitrile (20/80) as eluent (Figure 1b). The operating conditions and retention times of these sugars are shown in Table 2.

From these results, it appears that an effective separation of a mono-, di-, and tri-saccharide mixture is carried out in two stages :

1) Elution of monosaccharides with $CH_3CN/H_2O/CH_3COOH$ at 90/09/01; the retention times of di-, and tri-saccharides are long and their peaks too broad to be measured accurately.

2) Elution of di-, and tri-saccharides with CH_3CN/H_2O at 80/20 as mobile phase. The retention times of monosaccharides are very short; the resolution between their different peaks is poor.

In order to elute, over the same chromatogram, a mono-, di-, and tri-saccharides complex mixture, we considered using a flow-rate gradient.

The flow-rate gradient allows us to change the mobile phase flow-rate with time. Such possibilities are made possible by manipulating the ratio of water to acetonitrile in the eluent and programming the flow-rate. The elution of nine carbohydrates was programmed over a 20 min. period by using linear and concave gradient curves.

1) According to a linear flow profile; CH_3CN/H_2O are in the ratio 85/15. When the flow-rate is increased from 1 to 2 ml/min., we notice a poor separation of the first 3 monosaccharides (i.e., ribose, meso-erythritol, xylose) and good resolution for the di-, and tri-saccharides. The complete elution did not exceeded 32 minutes (Figure 3a).

2) With a concave flow profile; in this case, we modified only the flow-rate (from 1 to 2.5 ml/min.) and

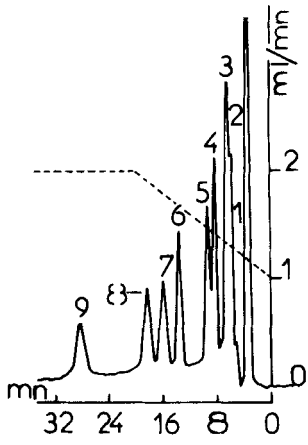

FIGURE 3a : Linear flow-gradient H_2O/CH_3CN (15/85) elution of 9 carbohydrates. 1.D(-)-ribose; 2. meso-erythritol; 3.D(+)-xylose; 4.D(-)-fructose; 5.D(+)-glucose; 6. sucrose; 7. maltose; 8.lactose; 9. raffinose.Conditions: flow-rate; from I to 2 ml/mn. over a 20 mn period. Injection amount : 0.2 mg of each of 9 sugars. Attenuation : 32mV.

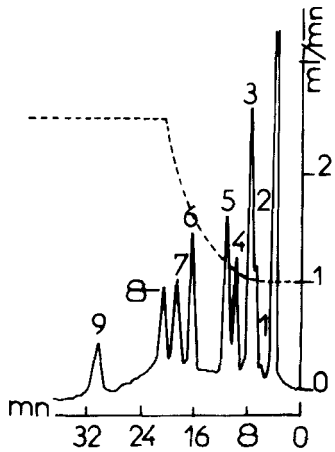

FIGURE 3b : Concave gradient flow-rate mode separation of 9 mono-, di-, and tri-saccharides. 1.D(-)-ribose (0.1 mg); 2. meso-erythritol (0.1 mg); 3.D(+)-xylose (0.2 mg); 4.D(-)-fructose (0.1 mg); 5.D(+)-glucose (0.2 mg); 6. sucrose (0.2 mg); 7. maltose (0.2 mg); 8. lactose (0.2 mg); 9. raffinose (0.2 mg). Conditions; solvent : H_2O/CH_3CN(15/85), flow-rate : from 1 to 2.5 ml/mn over a 20mn period, Attenuation : 32 mV.

the flow gradient profile. No detectable influence has been noticed with this approach (Figure 3b).

3) In a third stage, the polarity of the mobile phase was decreased (H_2O/CH_3CN, 10/90). The gradient was programmed with a linear scan and the period of flow-rate maintained unchanged. Under these conditions a good separation of 8 mono-, and di-saccharides was obtained. However, Raffinose, after one hour running, was still not eluted (Figure 4b).

4) With a linear flow profile, the composition of the eluent (H_2O/CH_3CN, 12.5/87.5) and the variation of flow-rate (from 0.8 to 2.5 ml/min.) were modified. No improvement was noted in relation to the above case Figure 4a).

5) To elute over a same chromatogram, the mine carbohydrates, several systems were investigated. The best separation was achieved under the following conditions : the gradient flow-rate system consisted of 13.5% water and 86.5% acetonitrile,programmed with concave scan from 1 to 2.5 ml/min. (Figure 5).

In conclusion, if a good separation of mono- and di-saccharides was desired, alternatives 3) or 4)might be the answer. For satisfactory resolution of di-, and tri-saccharides, the conditions given in 1) or 2) will be more appropriate. For an intermediate solution,case 5) will be the method of choice. However, the resolution

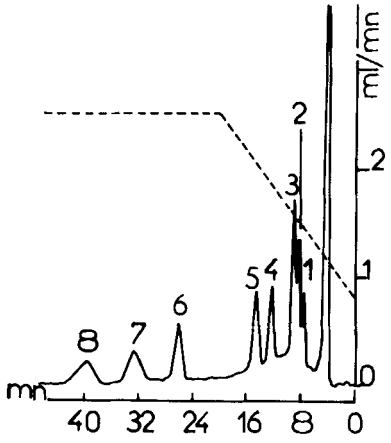

FIGURE 4a : Linear flow-gradient H_2O/CH_3CN(12.5/87.5) elution of 8 mono-, and di-saccharides. 1.D(-)-ribose (0.1 mg); 2. meso-erythritol (0.1 mg); 3.D(+)-xylose (0.2 mg);4.D(-)-fructose (0.1 mg); 5.D(+)-glucose(0.2 mg); 6. sucrose (0.2 mg); 7. maltose (0.2 mg); 8. lactose (0.2 mg). Conditions: 0.8 to 2.5 ml/mn over a 20 mn period. Attenuation: 32 mV.

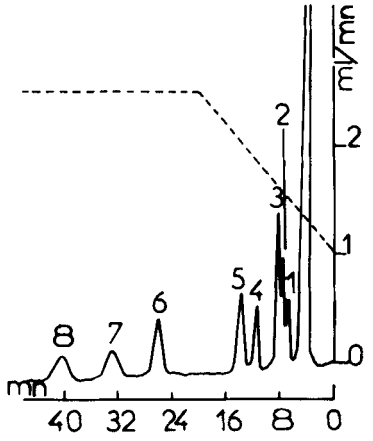

FIGURE 4b : Separation of 8 carbohydrates. Solvent : H_2O/CH_3CN(10/90), flow-rate : from 1 to 2.5 ml/mn over a 20mn period. Other conditions were as in Figure 4a.

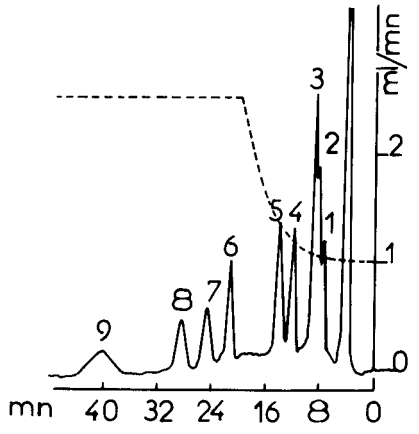

FIGURE 5 : Flow-gradient mode separation of 9 mono-, di-, and tri-saccharides with H_2O/CH_3CN (13.5/86.5). Other conditions were as in Figure 3b.

between the first three monosaccharides remains weak and the analysis time longer (45 min.).

To improve these two important parameters, and taking into account the retention times of different monosaccharides, we replaced in the studied carbohydrates mixture, ribose and meso-erythritol with two monosugars most commonly encountered in food-chemistry: rhamnose and arabinose. In a second stage, several operating conditions were considered to optimize the separation. With a linear gradient flow profile, the flow-rate was increased from 1 to 2 ml/min. over a 5 minute period. The eluting solvent being H_2O/CH_3CN in the ratio 15/85, a satisfactory separation of a mono-, di-, and tri-saccharides

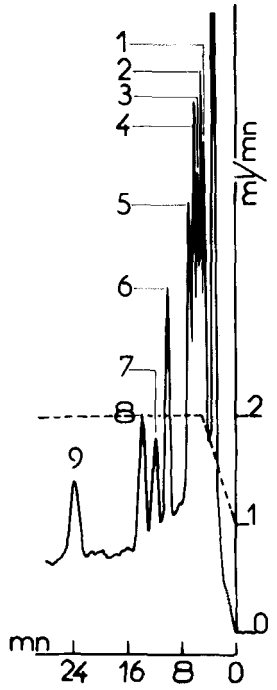

FIGURE 6 : Liear gradient flow elution profile of a synthetic carbohydrates mixture.1.L(+)-rhamnose; 2.D(+)-xylose; 3.L(+)-arabinose;4.D(-)-fructose; 5.D(+)-glucose;6.sucrose; 7.maltose; 8.lactose; 9.raffinose. Conditions;solvent : H_2O/CH_3CN(15/85),flow-rate: from 1 to 2ml/mn over a 5mn period, attenuation: 16mV,injection: 3 microliters of a mixture at 2%(w/v).

mixture was carried out within 28 min. Figure 6 shows that the 9 carbohydrates are eluted from the column in order of their molecular weights (i.e., mono-, di-, and tri-saccharides with the following sequence :

1) Pentoses (rhamnose, xylose, arabinose)

2) Hexoses (fructose, glucose)

TABLE 3

A - Resolution and HETP Calculated from Figure 5.

Carbohydrates	Resolution	Efficiency (plates/m)
Fructose Glucose	1.1	-
Sucrose	-	11000

B - Resolution and HETP Calculated from Figure 6.

Carbohydrates	Resolution	Efficiency (plates/m)
Fructose Glucose	0.6	-
Sucrose	-	5300

3) Disaccharides (sucrose, maltose, lactose)

4) Trisaccharides (raffinose).

To evaluate the degree of separation and efficiency of the column used, we compared the resolutions and HETP's obtained from chromatograms of figures 5 and 6. For the-

se calculations, three carbohydrates were considered : D(-)-fructose, D(+)-glucose, whose separation is often difficult, and sucrose, which gives a sharp, symmetrical peak. The results obtained are shown in Table 3.
As these tables indicate, if we want to obtain a satisfactory separation within 28 min. of a mono-, di-, and tri-saccharides mixture with an uniform chromatogram, the operating conditions of Figure 6 will be suitable . If, on the other hand, enhanced resolution and efficiency required for the hexoses and disaccharides, we would choose the chromatographic conditions of Figure 5.

QUANTITATIVE EVALUATION AND DETECTION LIMIT

Often, only very dilute solutions of sugars are available, so the detection limit for this system is therefore of interest. Figure 7 shows the calibration plots for five mono-, and four di-, and tri-saccharides which were established with reference solutions of different concentrations, using the RI detector. The operating conditions were those of Figure 6, with different attenuations. Standard solutions were prepared as follows : 100 mg of each of 9 sugars were mixed together and dissolved in 5 ml of distilled water.
0.2; 0.3; 0.5; 1; 3; 5 and 10 microliters of that solution were injected directly. The averages of peak heights (cm) from triplicate sample injections were plotted

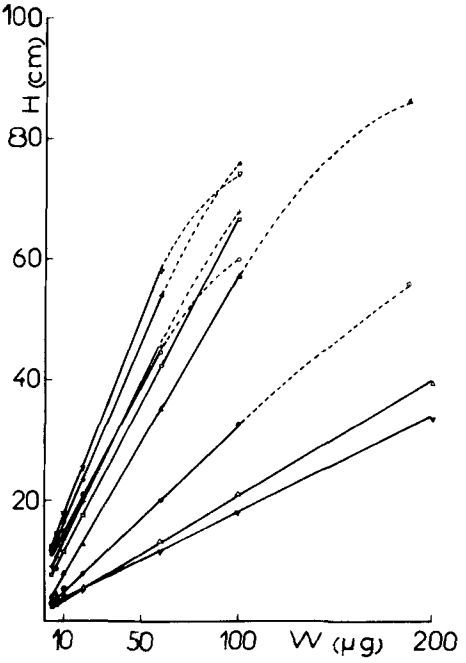

FIGURE 7 : Calibration curves. Peak height, H(cm)versus weight, W(µg) of injected carbohydrate.(▽)xylose; (●).fructose;(✚).rhamnose;(○).arabinose; (□).glucose;(▲).sucrose;(⊙).lactose;(△).maltose;(▼).raffinose. The chromatographic conditions were those of Figure 6.

against weight (microgram) of carbohydrates to obtain the standard curves. It can be seen that there are linear relationships between peak height and component weight in the investigated region from about :

- 4 to 60 micrograms for xylose, fructose, arabinose and rhamnose;
- 4 to 100 micrograms for glucose, sucrose and lactose;

- 4 to 200 micrograms for maltose;
- 6 to 200 micrograms for raffinose

for the injected samples.

The low detection limit for monosaccharides (4 micrograms), carried out with the Microbondapak NH_2 column and using a RI detector, is comparable to those obtained (2 to 12 micrograms) for the same underivatized monosaccharides on a chemical bonded amino stationary phase by Binder with UV detection at 188 nm (10).

APPLICATION

In order to further illustrate our above study, we determined the main carbohydrates found in commercial food products in Algeria. We chose two types of honey : one, which is widely distributed, the other was locally marketed variety. The honey samples were prepared according to the method of Thean and co-workers (15).The resultant solutions were analyzed with a Microbondapak NH_2 column under the experimental conditions outlined in Figure 6. The results showed that the aliquot from the first type of honey contained, primarily, four carbohydrates : fructose (35.6%), glucose (38.6%), sucrose(4%) and maltose (2.4%) -Figure 8a - ; while in the second aliquot , the major components were : fructose, glucose and sucrose, respectively, in the percentages : 21.9% ; 31.7% and 35.3%.

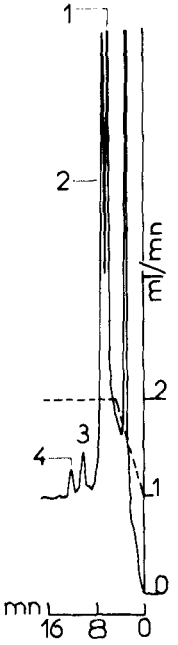

FIGURE 8a: Linear gradient flow elution profile of major components of commercial honey. 1.fructose; 2.glucose; 3.sucrose; 4.maltose. The analysis conditions were as in Figure 6.

It appears that the second type of honey is adulterated with a high sucrose content which reduces the levels of other sugars (Figure 8b). With regard to the first type of honey, the results found are comparable to those obtained by other authors for Orange Blossom Brand honey which was analyzed by HPLC (15) , and by the official methods of the AOAC (16).

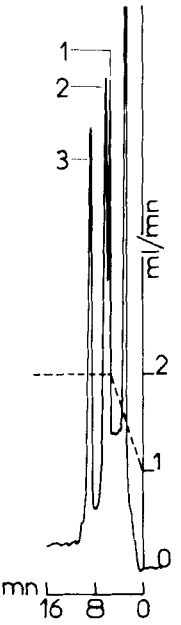

FIGURE 8b: Adulterated honey with a high sucrose content. 1. fructose; 2. glucose; 3. sucrose. The elution conditions were the same as in Figure 8a.

CONCLUSION

A high performance liquid chromatographic (HPLC) procedure based on a Microbondapak NH_2 column, an aqueous acetonitrile solvent and a gradient flow-rate mode has been developed. The method is rapid and no further treatment of the sample is necessary, except for the separation of undissolved solids contained in the solution. Depending upon the sugars' variety in the sample, many chromatographic conditions have been suggested

to obtain sufficient resolution and/or a satisfactory analysis time. If, for instance, raffinose is not present in the sample, only 16 minutes are necessary to elute an eight mono-, and di-saccharide mixture.Compared with the normally used detection by refractive index measurement, this system has the advantages of a lower detection limit and the possibility of shortening the analysis time for a complex mixture containing nine mono-, di-, and tri-saccharides. On the other hand, this procedure shows better performance than those employing UV detection for aqueous sugar mixture analysis without derivatization or pretreatment.

AKNOWLEDGEMENTS

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