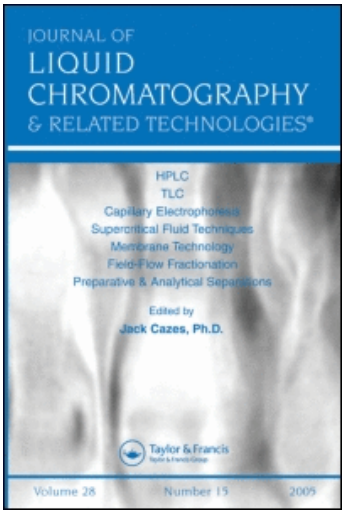

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ISOLATION AND DETERMINATION OF SUGARS IN *NICOTIANA TABACUM* ON AMINOPROPYL CHEMICALLY BONDED PHASE USING SPE AND HPLC

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ABSTRACT

Aminopropyl chemically bonded phases for solid-phase extraction (SPE) and high performace liquid chromatography (HPLC) have been prepared. Surface characteristics of packings before and after chemical modification were determined by means of different physico-chemical methods e.g. porosimetry, elemental analysis, CP/MAS NMR, chromatography, etc. The obtained packing materials were compared with commercially available packings (Silasorb NH_2 and LiChrosorb NH_2). Prepared columns were applied for the isolation and determination of sugars origined from dried leaves of the Nicotiana tabacum. The columns were characterized by high coverage density of aminopropyl phase and high sorption capacity gave good and reproducible separation results.

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INTRODUCTION

Chromatographic determination of different substances contained in natural material often causes some difficulties. This is connected with efficient and efficacious isolation of determined substances as well as with proper choice of the chromatographic conditions (**1**).

The isolation of different substances contained in biological materials requires usually the utilization of the liquid - solid extraction technique (solid phase extraction; SPE), using chemically bonded phases (CBP) as material packings (**2-4**). The packings with CBP used in SPE have wide applications due to the possibility of reproducible and multiple use, particularly in routin analysis. In this field the application of solvolytically stable material packings is fully substantiated and advisable (**5**).

It is known, from the literature that different producers recommend various material packings that unfortunately show different chromatographic properties from batch to batch. This relates especially to chemically modified packings containing aminopropyl groups. In contact with the aqueous mobile phase these materials quickly undergo hydrolytical destruction. Nevertheless, they are recommended for selective separation of saccharides (**1,5-8**).

In this paper we described NH_2 phases characterized by high coverage density ($\alpha_{NH2} > 4.0$ $\mu mol/m^2$) for HPLC and SPE. The packings obtained were used for isolation and determination of mono- and polysaccharides contained in dried leaves of the Nicotiana tabacum. Determination of

these substances is very important because it gives the possibility to control the fermentation processes of cigarette tobacco. Prepared packings have been compared with commercially available phases (LiChrosorb NH_2 and Silasorb NH_2). Surface characteristics of the applied materials were investigated by means of different physico-chemical methods.

EXPERIMENTAL

Apparatus

The parameters characterizing porosity, i.e.; specific surface area (S_{BET}), pore volume (V_p) and mean pore diameter (D) of the bare silica gels were determined by the low-temperature nitrogen adsorption-desorption method, using a Model 1800 Sorptomatic instrument (Carlo Erba, Milan, Italy).

The surface silanol concentration (α_{SiOH}) was determined by reacting the packings with dimethyl-zinc·tetrahydrofuran complex, according to the method proposed by Nondek and Vyskočíl (9). This reaction permits the determination of the amount of accesible surface silanol groups.

The surface concentration of the aminopropyl (α_{NH2}) ligands and the number of accesible NH_2 groups (n_{NH2}) per 100 Å^2 on the packing surfaces were calculated on the basis of carbon and nitrogen contents, determined with a Model 240 CHN analyser (Perkin-Elmer, Norwalk, CT, USA).

Solid-state NMR measurements were performed on a Bruker (Rheinstetten, FRG) MSL 200 spectrometer with samples of 200-300 mg in double-bearing rotors of zirconia. Magic angle spinning (MAS) was carried out at a spinning rate 4 KHz. ^{29}Si and ^{13}C cross-polarization (CP) MAS NMR spectra were recorded with a pulse length of 5 μs together with a contact time of 5 ms and a pulse repetition time of 2 s. All NMR spectra were externally referenced to liquid tetramethylsilane and the chemical shifts are given in parts per milion (ppm).

Chromatographic measurements were made using a liquid chromatograph consisting of an LC-20 piston pump (Pye Unicam, Cambridge, UK), a Model 7120 injection valve (Rheodyne, Berkeley, CA, USA) equipped with 10 μl injection loop, a Model RIDK 101 differential refractometer (Laboratorni pristroje, Praha, Czechoslovakia) and a TZ-4100 linear recorder (Laboratorni pristroje).

Materials and reagents

Following silica gels were used to preparation of amino (NH_2) chemically bonded phases (CBPs): SG-7/G (Polymer Institute of the Slovak Academy of Sciences, Bratislava, Chechoslovakia)(**10,11**), LiChrosorb Si-100 and Kieselgel Si-60 (E. Merck, Darmstadt, FRG). Physico-chemical properties of the bare silica gels are listed in Table 1.

This table gives additional information of surface characteristics of other unmodified supports (Silasorb; Lachema, Brno, Chechoslovakia and LiChrosorb Si-100; Merck) used by producers to prepare commercial phases with NH_2 groups.

Table 1. Physico-chemical characteristics of bare silica gels used as the support for $-NH_2$ CBP applied in HPLC and SPE, where: d_p - mean particle diameter (μm); S_{BET} - specific surface area (m^2/g); D - mean pore diameter (nm); V_p - pore volume (cm^3/g); α_{SiOH}-the number of acessible silanol groups per 100 Å^2 of silica gel supports.

No of packing	Type of packing	d_p	S_{BET}	D	V_p	α_{SiOH}
1.	SG-7/G	7	361	20.0	2.10	3.13
2.	LiChrosorb Si-100	7	246	12.1	1.19	2.68
3.	LiChrosorb Si-100[a)]	10	260	13.2	1.21	2.72
4.	Silasorb[a)]	10	300	15.3	1.32	2.56
5.	Kieselgel Si-60[b)]	30-63	335	8.6	0.69	-

a) commercial bare silica gels used by producers as supports for the preparation of NH_2 phases,

b) silica gel used for the preparation of SPE packing.

3-aminopropyltriethoxysilane (NH_2) (Fluka, Buchs, Switzerland) was used for chemical modification. All solvents, e.g., toluene, benzene, hexane, methanol, acetonitrile, dioxane and water (Merck, Darmstadt) were of analytical-reagent grade.

Stainless-steel tubes (100 x 4 mm I.D.) were purchased from Reagents Chemical Factory-Odczynniki Chemiczne (Lublin, Poland).

Chemical bonding procedure

The chemical modifications were carried out in glass reactor under a nitrogen atmosphere, carefully avoiding moisture (**12**). 5 g portions of silica gels were dried

Table 2. Characterization of packings after chemical modification by aminopropylsilane where: HM-home made; CP-commercial product; P_C-percent of carbon (%); P_N-percent of nitrogen (%); α_{NH2}-concentration of aminosilyl groups on the surface (μmol/m^2); n_{NH2}-the number of accesible NH_2 groups per 100 Å^2 of silica gel surface.

No.	Type of phase	Origined from	Coverage			
			P_C	P_N	α_{NH2}	n_{NH2}
1.	SG-7/G-NH_2	HM	5.2	1.6	4.45	2.736
2.	LiChrosorb Si-100 NH_2	HM	3.5	1.3	4.43	2.651
3.	LiChrosorb NH_2	CP	3.1	1.1	3.63	1.997
4.	Silasorb NH_2	CP	2.7	0.9	2.86	1.745
5.	Kieselgel NH_2	HM	5.3	1.5	4.79	2.882

at 180°C under vacumm (10^{-3} Pa) for 12 h. Then, the samples were reacted with 3-aminopropyltriethoxysilane and heated to 110 ± 5°C for 8 h. The product was filtered off and washed with dry toluene, methanol, n-hexane, and dried at room temperature.

The elemental analysis data of the prepared and commercial packing materials with aminopropyl groups are listed in Table 2.

HPLC column packings and SPE columns preparation

A slurry of 2 g of the prepared and the commercial phases dispersed in 35 ml of dioxane-methanol (2:5 v/v) is placed in an ultrasonic bath for 5 min and then filled into the HPLC column using 100 ml acetonitrile as a packing solvent. All HPLC columns were packed with a Haskel (Burbank, CA, USA) packing pump under a constant pressure of 45 MPa.

The SPE columns were prepared by packing 2 ml plastic extraction tubes with the appropriate material to a bed height of 2 cm.

Natural samples preparation

0.5 g pulverized leaves of tobacco have been extracted with 2 ml water and 2 ml acetonitrile and placed for 5 min in an ultrasonic bath. After this procedure 46 ml of acetonitrile were added and the obtained suspension was cleaned on two SPE columns connected in series. These columns were packed with bare silica gel and silica gel with C_{18} phase. For isolation and preconcentration of the determined sugars the SPE column with bonded NH_2 phase has been used.

RESULTS AND DISCUSSION

Packings characterization

Table 1 lists the physico-chemical characteristics of bare silica-gels used as a support for chemical modifications with 3-aminopropyltrietoxysilane. These material packings were selected in such a way that it could state whether the elaborated method of modification of the surface leads in fact to packings characterized by a so called dense coverage (**14**). Hence, our investigations have shown that the adsorbents with a greater pore volume and pore diameter show higher sorption capacities as well as a better mass transfer. Previously it has been reported (**10**), that the SG-7/G material (Table 1) characterized by a great number of accesible surface silanol groups (α_{SiOH} = 5.21 μmol/m^2). This fact has a great importance during the formation of CBPs.

Comparing the percentages of carbon (P_C) and nitrogen (P_N) (Table 2) determined by means of elemental analysis and recalculating these quantities (**14**) on α_{NH2} and n_{NH2} values it can be seen that the highest coverages with trifunctional aminopropyl ligands were obtained in the case of two packings i.e. SG-7/G (packing 1, Table 2) and LiChrosorb Si-100 modified in our laboratory (packing 2, Table 2). The α_{NH2} and n_{NH2} values obtained for these two packings prepared on the basis of LiChrosorb (packings 2 & 3) (LiChrosorb NH_2 is a commercial packing) show significant differences between them (at about 20 %) although being modified with the same modifier (silane). This may explane the dissimilarity of the course of the chemical modification as well as the differences in the physico-chemical properties of the bare silica gels used as the supports (see Table 1). The parameters characterizing Silasorb NH_2 after chemical modification are worse (Table 2), although this adsorbent fullfils all demands made for the CBP supports (**11,13**).

SPE material prepared on the basis of Kieselgel Si-60 (packing 5, Table 2) was characterized by very good coverage parameters. The results obtained for the packings 1, 2 and 5 (Table 2) are not surprising regarding that a trifunctional silane with a short chain length was used for modification (**4**). Application of a trifunctional silane leads to the formation of net-polymer CBP structures on the support surface (**14**). This conclusion is confirmed by the ^{29}Si CP MAS/NMR spectra of bare SG-7/G (Fig 1 a) and the same support modified with aminopropyl silane (Fig 1 b).

The analysis of these spectra (Fig 1 a & b) results that chemical blocking of surface silanol groups (Q_2 -

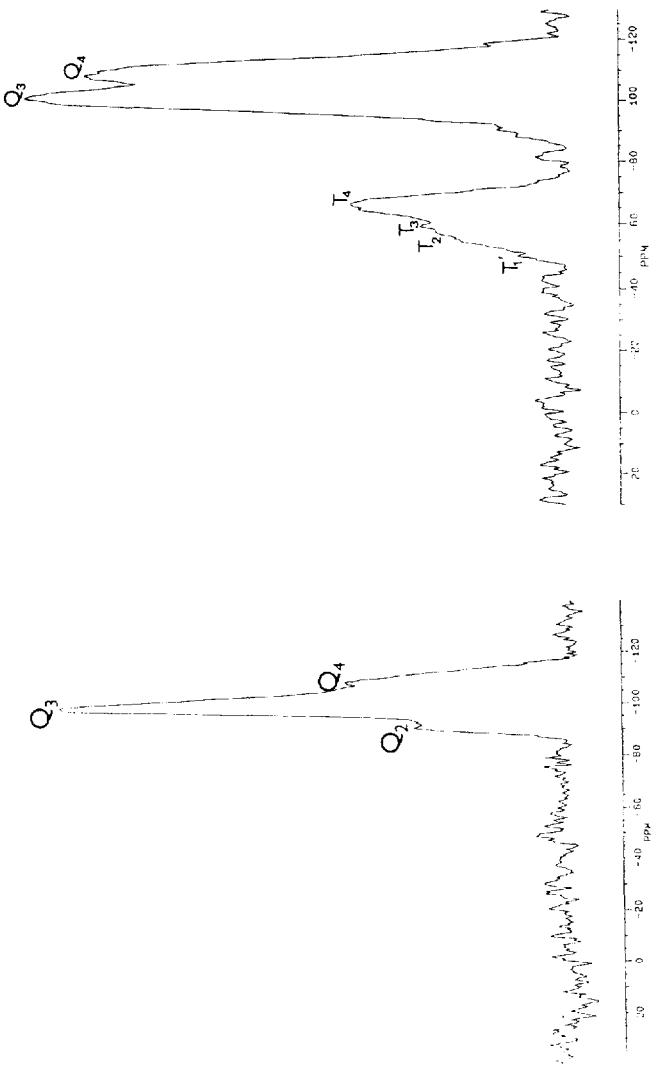


Fig. 1. ^{29}Si CP/MAS NMR spectra of unmodified (a) and modified (b) SG-7/G silica.

geminal, δ = -91 ppm; Q_3 - free, δ = -100 ppm and Q_4 - siloxane, δ = -108 ppm) leads to a decrease of the contribution of Q_2 and Q_3 groups. The increase of the contribution of siloxane groups (Q_4) as a consequence of blocking of the free and geminal groups by modifier molecules is observed (**14-17**). T_1, T_2, T_3 and T_4 peaks are registrated at a chemical shift ranged from δ = -49 to -66 ppm (Fig. 2) correspond to bonded aminosilyl phase (**14-17**).

The behaviour of bonded aminopropl ligands is better characterized by the ^{13}C CP/MAS NMR spectrum in Fig 3.

This spectrum shows major peaks at +60 and +17 ppm, corresponding to the CH_2 and CH_3 carbons of unreacted ethoxy groups. Peaks at +43, +27 - +21, and +10 ppm, attributed to the C-1, C-2 and C-3 carbons (**15-17**). Additional peaks are assigned to different species e.g. to ethanol and water molecules condensed on silanol groups adsorbed and desorbed from these groups or/and to formation of intermediate interface forms of amino group containing the hydrogen bonds (Fig. 4)(**16-18**).

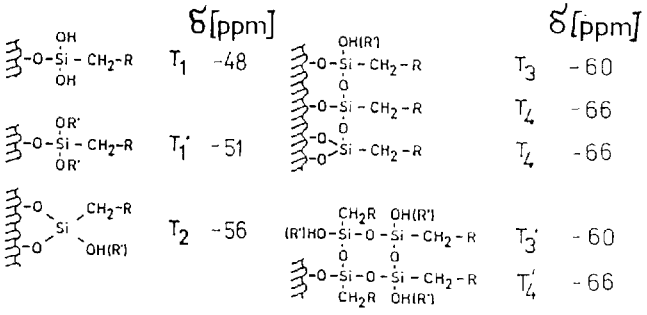
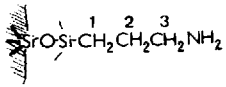


Fig. 2. Possible surface structure on the siliceous support after modification with trifunctional silane (T).



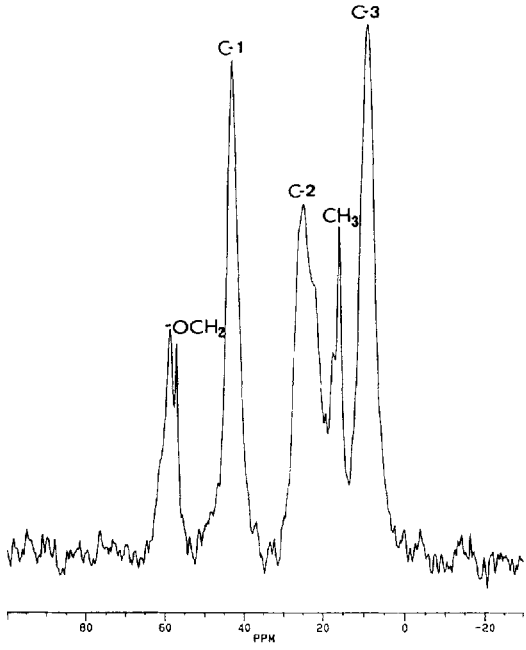


Fig.3. ^{13}C CP/MAS NMR spectrum of modified SG-7/G silica with 3-aminopropyltriethoxysilane.

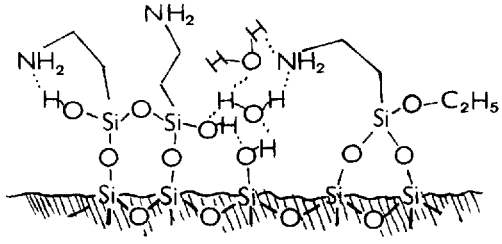


Fig 4. Possible structural interactions for amino-derivatized silica gel.

This effect can influence on the rotation of the chromatographed substances especially in the case of low coverages (greater contribution of residual silanol groups).

HPLC investigations

Chromatographic estimation of prepared packings (packing 1 & 2) and their comparison to commercial materials (packing 3 & 4) were made on the basis of the results of the separation of test mixture of mono- and disaccharides (Fig 5). In Table 3 are listed also the capacity factors (k') and resolution values (R_S) for worset separated compounds (arabinose and fructose).

From the comoarison of the chromatograms and data listed in Table 3 results that regarding the separation selectivity the best resolution of the analyzed sugars is obtained with the column packed with SG-7/G NH_2 (column A, Table 3).

The worst results were obtained on the column packed with commercial material packing, Silasorb NH_2 (packing

Table 3. k'and R_S values obtained for the fructose and arabinose on the estimated columns using RI detector. Mobile phase: 80-20 % v/v ACN-H_2O, flow rate - 1ml/min.

Peaks No.	Column Solut	A k'	A R_S	B k'	B R_S	C k'	C R_S	D k'	D R_S
2.	Arabinose	2.60		2.24		2.09		2.38	
			1.52		1.4		1.32		0.93
3.	Fructose	3.20		2.70		2.55		3.10	

where:
A - SG-7/G NH_2; B - LiChrosorb Si-100 NH_2;
D - LiChrosorb NH_2; D - Silasorb NH_2.

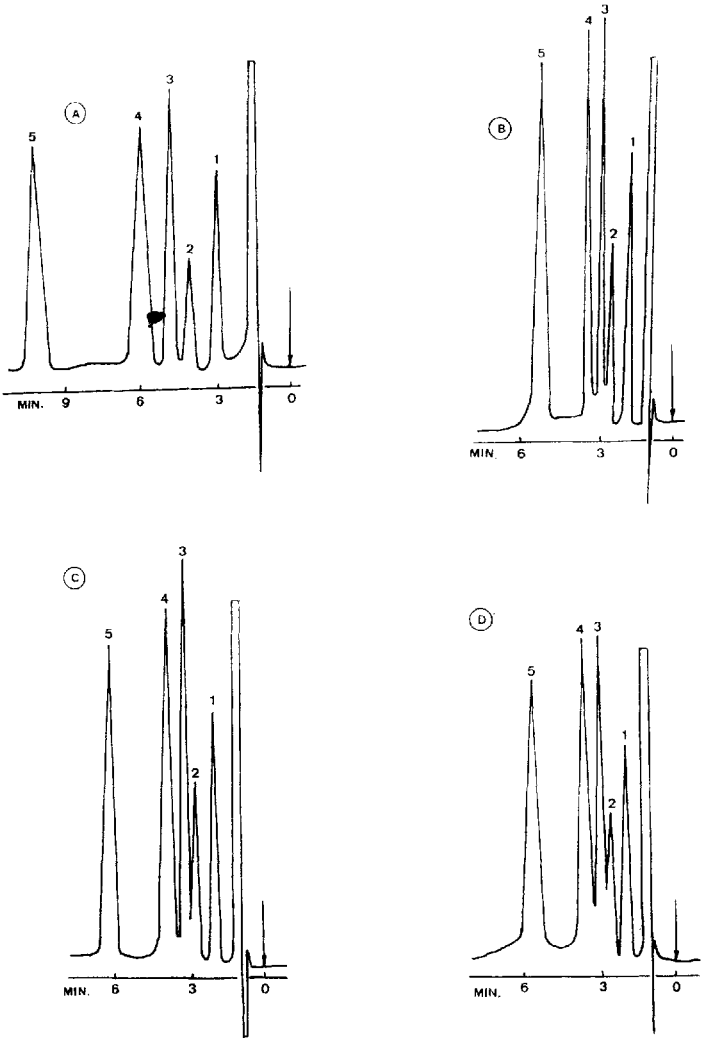


Fig. 5. Separation of some saccharides mixture obtained on the modified column packings: A - SG-7/G NH_2; B - LiChrosorb Si-100 NH_2; C - LiChrosorb NH_2^R; D - Silasorb NH_2. Chromatographic conditions: peaks: 1) ramnose, 2) arabinose, 3) fructose, 4) glucose, 5) saccharose; mobile phase: acetonitrile-water 80-20%; flow rate: 1 ml/min; detector: RI.

D, Table 3). Undoubtely, the coverage density of aminopropyl groups (Table 2) and its influence on the retention data of chromatographed substances takes then place (**19,20**). It suggests that the resolution improves with an increase of α_{NH2} and /or n_{NH2} values. In consequence, the separation selectivity may be changed not only by the choose of an appropriate composition of the mobile phase but also by an appropriate choose of a defined coverage density of the packing material (**14,22**).

In this case, the effect of the porosity does not play probably an important role (**11,13,21**). From the comparison of the packings prepared on the basis of LiChrosorbs (packing 2 & 3) results that the better resolution is obtained on the material synthezised according to our procedure (Fig. 5, Table 3).

Table 4. Recovery values for standards (RS) and sugars isolated from Nicotiana tabacum (RR) as well as standard deviation (SD).

Determined solute	Recovery (%) RS[a)]	SD	RR[b)]
Ramnose	40	± 6.47	-
Arabinose	50	± 5.81	-
Fructose	68	± 3.87	15.0
Glucose	80	± 3.03	2.4
Saccharose	91	± 2.54	1.1

a) standards concentration 10 mg/ml
b) concentration of sugars in mg/g of tabacco.

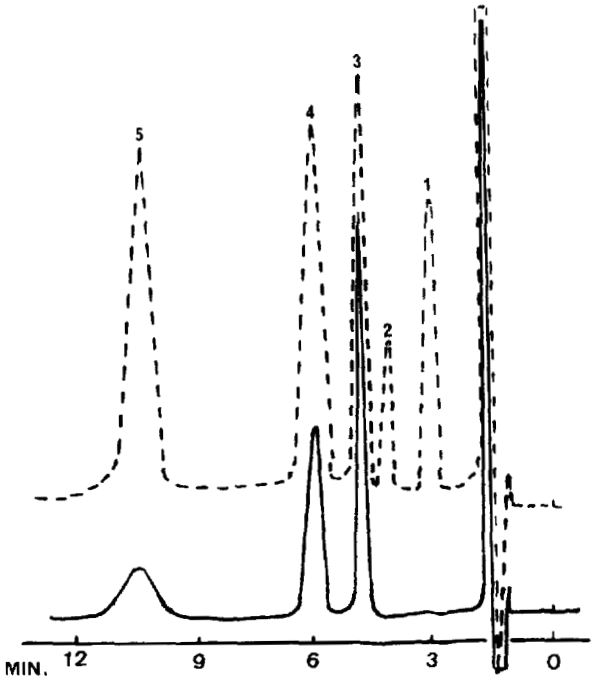


Fig. 6. Chromatograms of sugers separated on the column with SG-7/G NH_2. Continous line - separation of sugers standard mixture, dashed line - separation of sugers isolated by SPE detected in tobacco. Separation condition see experimental part and Fig. 5.

SPE and HPLC applications

Qualitative and quantitative determination of carbohydrates contained in Nicotiana tabacum used for production of cigarettes was carried out using off-line clean-up solid phase extraction (SPE) column with chemically bonded NH_2 phase on the basis of Kieselgel Si-60 (packing 5, Tables 1 & 2).

Using the procedure described in the experimental part and considering the simultaneous isolation of five components of a standard mixture of sugars (Table 4) we have obtained recoveries in the range of 40 to 91 % (standard deviation, SD, from ± 6.47 to ± 2.54).

In the leaves of the Nicotiana tabacum we identified only three from five expected sugars. The highest level was observed for fructose (15 mg/g tobacco) (Fig. 6, Table 4). Moreover, it appeared that the application of SPE column with chemically bonded NH_2 phase allows to 25 fold the concentration of the isolated carbohydrates. The detection level of these substances, using a RI detector, depends on the type of the determined substance and varies from 0.4 to 1.0 ng/ml.

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